# THE APPLICATION OF MOLECULAR TECHNIQUES FOR THE RAPID AND SENSITIVE DETECTION OF GASTROINTESTINAL PATHOGENS DIRECTLY IN FOOD

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## DECLARATION

I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of Doctor of Philosophy being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised another's work

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Andrew Westby……
Jim McLauchlin……

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## ABSTRACT

Conventional microbiological methods are slow, labour intensive and are unable to meet the demands for rapid food testing. Molecular methods, such as PCR, offer a rapid, sensitive and specific means of detecting pathogens, however loss of sensitivity and lack of robustness have been reported when PCR is applied to heterogeneous and complex food matrices. The aim of this study was to establish a rapid, reliable and sensitive molecular method to detect pathogens in food samples.

Real-time PCR assays for the detection of *Campylobacter jejuni* and *coli*, *Clostridium perfringens*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* in food enrichment samples were developed. A novel organism was constructed using a *gfp* gene cloned into the chromosome of a non-pathogenic *Escherichia coli*. Viable cells of the modified strain were encapsulated in Lenticule discs and used as process control in the PCR assays. MagNA Pure™ automated extraction was shown to be robust and reliable for preparing bacterial DNA from food enrichment broths. The PCR assays and MagNA Pure™ was applied to enrichment broths inoculated with 558 naturally-contaminated food and environmental samples in a field trial. Concordance was found between PCR results and those obtained using standard culture methods. Loss of assay sensitivity or PCR inhibition was detected in 6 % (32) of the enrichment samples. To improve the sensitivity the *L monocytogenes hlyA* gene PCR was nested. The assay was applied for the sensitive non-cultural diagnosis of listeriosis, with *L monocytogenes* detected in 15 of 17 clinical samples from patients with suspected listeriosis.

In conclusion, these assays provided a high throughput, robust, reliable PCR detection methods that could be used in clinical and food testing laboratories. The methods will be essential in outbreak situations and could be further developed to detecting bacterial pathogens, viruses, parasites, new and emerging pathogens.

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## ABBREVIATIONS

| | |
|---|---|
| ACC | Aerobic colony count |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pairs |
| CFU | Colony forming unit |
| CSF | Cerebral spinal fluid |
| $C_T$ | Cycle threshold |
| $\Delta R_n$ | Normalised reporter signal |
| DNA | Deoxyribonucleic acid |
| EMA | Ethidium monoazide |
| EC | European Commission |
| PC | Internal control |
| IPC | Internal positive control |
| HPA | Health Protection Agency |
| µl | Microlitre |
| ml | Millilitre |
| min | Minute |
| NCTC | National Collection of Type Cultures |
| N/a | Not applicable |
| ND | Not detected |
| N/t | Not tested |
| NASBA | Nucleic acid sequenced based amplification |
| PCR | Polymerase chain reaction |
| RT PCR | Reverse transcriptase PCR |
| s | Second |
| SD | Standard deviation |
| *Taq* | Thermus aquaticus |
| $T_m$ | Melting temperature |
| U | Units |
| v:v | Volume to volume |

# CHAPTER 1

INTRODUCTION

## 1.1 Foodborne disease

Foodborne disease was defined by the World Health Organisation (WHO) as 'any disease of an infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water' (WHO, 2007). The WHO estimated that in 2005 1.5 million people died worldwide from diarrheal diseases (Buzby and Roberts, 2009). In industrialized countries, the percentage of the population suffering from foodborne diseases each year has been reported to be up to 30% (WHO, 2007). Organisms that cause foodborne disease include bacteria, viruses, parasites, prions, algae and fungi (Tauxe, 2002). Humans acquire these pathogens through a number of routes including eating contaminated food, contact with animals and contact with a contaminated environment (Pires *et al.,* 2009). Foodborne transmission is recognized as being responsible for a major proportion of these infections, and foodborne disease may involve many different food sources and commodities (Pires *et al.,* 2009).

### 1.1.1 Pathogenesis of food poisoning bacteria

Bacterial food-borne illnesses can be classed in two main groups: gastrointestinal infections, leading to proliferation of pathogenic microbes in the infected host for example, salmonellosis, and toxin induced food poisoning, due to the presence of a preformed toxin resulting from bacterial growth for example, *B. cereus* in the food (Abubakar *et al.,* 2007). An overview of the aetiological features of bacterial food poisoning is given in Table 1.1. The symptom that is typical of bacterial foodborne illness is diarrhoea. Diarrhoea caused by pathogenic bacteria results from an imbalance of absorption and secretion of ions and solute across the gut epithelium, followed by the movement of water in an attempt to restore the appropriate ion concentrations (Viswanathan *et al.,* 2009). The imbalance is often caused by the presence of bacteria that secrete toxins that disturb the organisation of the epithelium (Viswanathan *et al.,* 2009).

**Table 1.1 Symptoms of foodborne bacterial illnesses.**

| Causative organism | Onset of symptoms | Clinical symptoms |
|---|---|---|
| *Bacillus cereus* | Diarrhoeal syndrome ranges 8 to 16 hours. Emetic syndrome ranges 1 to 5 hours. | Two distinct syndromes: diarrhoeal and emetic. Diarrhoeal syndrome produces acute diarrhoea and abdominal pain. Emetic syndrome produces acute nausea, vomiting and stomach cramps and diarrhoea may occur later |
| *Campylobacter* spp. | 2 to 11 days | Prodromal fever and malaise, followed by abdominal pain and profuse diarrhoea |
| *Clostridium botulinum* | 24 to 72 hours | Fatigue, lassitude, dizziness and effects on the central nervous system including speech difficulties and visual disturbances leading to flaccid paralysis |
| *Clostridium perfringens* | 8 to 22 hours | Abdominal pain, nausea and diarrhoea, with vomiting rare |
| *Escherichia coli* | | Symptoms vary according to the type of *E. coli* infection |
| *Listeria monocytogenes* | Onset unknown but probably exceeds 12 hours to 3 months | Flu-like symptoms, gastrointestinal symptoms, meningitis, abortion in pregnant females and septicaemia. |
| *Salmonella* spp. | 12 to 48 hours | Diarrhoea, vomiting, fever and malaise |
| *Shigella* spp. | 12 to 50 hours | Diarrhoea with mucoid, bloody stools |
| *Staphylococcus aureus* | 2 to 6 hours | Vomiting, abdominal pain and diarrhoea on some occasions. |
| *Vibrio parahaemolyticus* | 2 to 48 hours | Profuse diarrhoea that leads to dehydration, vomiting and fever. |
| *Yersinia enterocolitica* | 24 to 48 hours (possibly longer) | Abdominal pain, fever, headache, diarrhoea and possibly vomiting and malaise |

Adapted from Roberts *et al.* (1995).

Generally, foodborne disease is a mild self-limiting illness, however occasionally, foodborne infections may progress to more severe conditions (Abubakar *et al.,* 2007). For example, the verocytotoxigenic *Escherichia coli* serotype O157:H7 can cause haemolytic uremic syndrome and *Campylobacter*

*jejuni* can cause reactive arthritis and Guillain–Barré syndrome (Abubakar *et al.,* 2007). *Listeria monocytogenes* can cause meningitis, meningoencephalitis, and septicaemia (Vazquez-Boland *et al.,* 2001). In addition *L. monocytogenes* infection in pregnant women can lead to an intrauterine infection resulting in stillbirths and miscarriages (Vazquez-Boland *et al.,* 2001).

### 1.1.2 **Cases of foodborne illness in England and Wales**

The Food Standards Agency (FSA) identified *Salmonella*, *Campylobacter*, verocytotoxin producing *E. coli* O157:H7, *Listeria monocytogenes* and *Clostridium perfringens* as the bacteria that cause either significant numbers of cases of foodborne illness or those which cause severe disease, or both (FSA, 2007). In 2005, these bacteria were ranked by the FSA in terms of causes of deaths, hospitalisations and total number of cases (Table 1.2). *Campylobacter* spp. ranked first in hospitalisations and total number of cases, *E. coli* O157:H7 ranked third for hospitalisations and total number of cases, non-Typhoidal Salmonellas ranked second in all three categories. *L. monocytogenes* ranked highest for cause of deaths. *Salmonella* is a major cause of foodborne infection in England and Wales with 37,298 cases of human infection confirmed by the Health Protection Agency (HPA) Laboratory of Enteric Pathogens from 2004 to 2006 (Kafatos *et al.,* 2008).

**Table 1.2 Bacterial causes of death and hospitalisation by foodborne illness in 2005.**

| Pathogen | Deaths | | Hospitalisations | | Total cases | |
|---|---|---|---|---|---|---|
| | number | ranking | number | ranking | number | ranking |
| *Campylobacter* spp. | 70 | 3rd | 13,930 | 1st | 295,500 | 1st |
| *E. coli* O157:H7 | 20 | | 400 | 3rd | 1,100 | 3rd |
| *L. monocytogenes* | 130 | 1st | 380 | | 400 | |
| *Salmonellae* (non-typhoidal) | 100 | 2nd | 1,220 | 2nd | 33,400 | 2nd |

Taken from: http://www.food.gov.uk/multimedia/pdfs/board/fsa071005a.pdf

### 1.1.3 **Under reporting of foodborne illness in England and Wales**

In England and Wales all Doctor's surgeries have a statutory duty to notify a Proper Officer of any suspected cases of food poisoning (Sethi *et al.,* 1999) under the Public Health (Infectious Diseases) Regulations 1988 (HPA, 2009). The Health Protection Agency (HPA) collates the numbers of cases of food poisoning, which is used to identify possible outbreaks, to trigger investigations and enable legal measures to be taken when necessary to control infection. Food poisoning account for the largest number of statutory notifications of illness with 72,382 cases reported in 2007, the next highest being 7,196 cases of mumps (HPA, 2009). The number of cases of food poisoning in England and Wales peaked in 1997 at 93,932 cases notified, yet has gradually decreased since then (HPA, 2009).

Most cases of food poisoning go unreported, because to be registered the individual must provide their general practitioner with a specimen for analysis and the laboratory must identify the pathogen and report the result (Abubakar *et al.,* 2007). The HPA has estimated that only one in 136 cases of food poisoning is reported (Anon., 2003). The cause of infectious intestinal disease is often not identified due to the difficulties described above and the percentage of cases where illness is of unknown aetiology is approximately 48% (Adak *et al.,* 2002).

### 1.1.4 **Increase of food poisoning cases in the UK**

The incidences of food poisoning steadily increased through the 1980s and 1990s in the UK; by 1995, it was estimated that there were 10.5 million cases of infectious intestinal disease in the UK, with 2.4 million of these directly attributed to the consumption of food (Adak *et al.,* 2002). At this time foodborne disease was estimated to cost the UK over £750 million per year (Adak *et al.,* 2002). In this period, the social pattern of eating has changed

from home-prepared food to increasing use of pre-prepared dishes and consumption of fresh produce, in addition more people are eating at commercial food service establishments (DuPont, 2007). The risk of foodborne illness has increased with the increased availability of fresh foods and when food is eaten in public restaurants (DuPont, 2007).

### 1.1.5 **Incidence of foodborne illness**

Determining that an illness is foodborne can be problematic if symptoms do not develop for several days or, in the case of Listeriosis up to several weeks (Vazquez-Boland *et al.,* 2001). In the case of Listeriosis, monitoring incidence and surveillance of foods can provide information about this pathogen and its effects within the population. Since 2000, there has been an increase in the number of Listeriosis cases reported in England and Wales with an average of 109 cases per year between 1990 and 2000 rising to 185 per year cases between 2001 and 2006 and peaking at 230 per year in 2007 (HPA, 2009). Annual increasing incidence of Listeriosis has also been reported in Belgium, Denmark, Finland, Germany, Netherlands, Sweden and Switzerland since 2000 (Goulet *et al.,* 2008). There appears to be no underlying cause that has been attributed to the rise in cases, however many cases appear to be among people over 60 years of age (Goulet *et al.,* 2008).

In addition to the increase in sporadic cases in the UK since 2000, there were also four outbreaks of *L. monocytogenes* infection associated with sandwiches provided in hospitals from 1999 to 2004 (Little *et al.,* 2008). Surveillance of food products including food surveys have been employed to indicate the microbiological quality of food on the market. A survey of cooked sliced meat performed by the HPA in 2008 revealed the contamination of ready-to-eat pork ears and tongue roll imported from Lithuania with potentially hazardous levels of *L. monocytogenes* (FSA, 2009).

### 1.1.6 **Outbreaks of food poisoning**

A food-borne outbreak is defined as an occurrence of two or more cases of a similar illness resulting from the ingestion of a common food (Abubakar *et al.,* 2007). Food poisoning outbreaks are often recognised due to a defined cluster of cases of food poisoning that can be attributed to the consumption of a particular meal or food item and can be determined by epidemiological and microbiological investigations. For example, a national outbreak of *S. enterica* Montevideo in chocolate bars occurred in the UK in 2006 (Elson, 2006a). Between March and June 2006, the HPA received 59 isolates of *S. enterica* Montevideo from human cases of infection in England and Wales, which exceeds the baseline level of cases expected for this unusual serotype of *Salmonella*.

The increased number of these cases of infection caused epidemiological investigations through Enter-net (an international surveillance network for human gastrointestinal infections) and microbiological typing. Pulse field gel electrophoresis of the isolates established that they were indistinguishable and there was probably as common link (Elson, 2006b). The isolates were designated an outbreak type (*Smvd*X07) which was linked by retrospective examination to nine *Salmonella* isolates from anonymous food samples. The information was relayed to the FSA on 23$^{rd}$ June 2006 and seven types of confectionary products were recalled due to the potential contamination with *S. enterica* Montevideo and an alert was placed on RASFF (Elson, 2006a).

Cadbury Schweppes issued a press release outlining the recall and stated that the products contained minute traces of *Salmonella*. As part of the investigation, the Advisory Committee on the Microbiological Safety of Food (ACMSF) stated that the presence of *Salmonella* in chocolate was unacceptable. Cadburys did not have a modern risk assessment for *Salmonella* and the end-point testing used was not a suitable for guaranteeing the safety of

food. The method used, most probable number (MPN), was unsuitable for chocolate testing because sample heterogeneity, including clumping of bacteria, would underestimate the level and likelihood of *Salmonella* contamination. The outbreak resulted in 42 cases, including three hospitalisations and a fine of £1 million after Cadburys pleaded guilty to nine food safety offences. Cadburys had altered its zero tolerance policy regarding *Salmonella* in its products, which was regarded as a serious case of negligence.

### 1.1.7 **Legislation for the provision of food that is fit to consume**

In the UK it is the Food Standards Agency's responsibility to ensure the provision of safe food for consumption in accordance to UK Food Safety Act (Amendment) Regulations 2004 SI No. 22900, the General Food regulations 2004 SI No. 3279 and European Union Food Law (Regulation (EC) No 178/2002). The EC Food Law requires that 'food shall not be placed on the market if it is unsafe, and food shall be deemed to be unsafe, if it is considered to be injurious to health or unfit for human consumption' (EC, 2002).

As part of this law, the European Commission issued the microbiological criteria for foodstuffs that are used for monitoring and enforcing compliance of businesses to food law. Food businesses should primarily monitor their provision of safe food using good hygiene practices, good manufacturing practices and the operation of hazard analysis critical control points (HACCP) (Lynch *et al.,* 2009). HACCP can be monitored using the Microbiological Criteria that stipulate unacceptable levels of *Salmonella*, *Listeria monocytogenes*, *Escherichia coli* and *Cronobacter sakazakii* in various foods (EC, 2007). The Microbiological Criteria are particularly important when considering new methods for the detection of foodborne pathogens, because the criteria stipulate the volume of food and the analytical method required. Table 1.3 shows the Microbiological Criteria for microorganisms, their toxins or metabolites in a food when the food product is on the market and during its

shelf life. The Microbiological Criteria require that International Organization for Standardization (ISO) methods are used to assess the microbiological quality of imported foods and when the results of food analysis are used to prosecute a food business through enforcement of the UK Food Safety Act.

**Table 1.3 The European Commission food safety criteria for products placed on the market during their shelf life.**

| Pathogen | Analytical method | Food type | Limits and number of samples to be tested |
|---|---|---|---|
| *L. monocytogenes* | EN/ISO 11290-1 | RTE foods intended for infants and ready-to eat foods for special medical purposes | Absence in 25g (10 samples) |
| | EN/ISO 11290-2 | RTE foods able to support the growth of *L. monocytogenes,* other than those intended for infants and for special medical purposes[1] | 100 cfu/g (5 samples) |
| | | RTE foods unable to support the growth of *L. monocytogenes,* other than those intended for infants and for special medical purposes | 100 cfu/g (5 samples) |
| *Salmonella* | EN/ISO 6579 | Minced meat and meat preparations intended to be eaten raw; gelatine and collagen; cheeses, butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation; milk powder and whey powder; meat products intended to be eaten raw, ice cream, egg products and RTE foods containing raw egg, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk; cooked crustaceans and molluscan shellfish; live bivalve molluscs and live echinoderms, tunicates and gastropods; RTE sprouted seeds, pre-cut fruit and vegetables, unpasteurised fruit and vegetable juices. | Absence in 25g (5 samples) |
| | | Dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age | Absence in 25g (30 samples) |
| | | Minced meat and meat preparations made from poultry meat or other species than poultry intended to be eaten cooked; mechanically separated meat; | Absence in 10g (5 samples) |
| Staphylococcal enterotoxins | CRL method for milk | Cheeses, milk powder and whey powder | Not detected in 25g (5 samples) |
| *Enterobacter sakazakii* | ISO/DTS 22964 | Dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age | Absence in 10g (30 samples) |
| *E. coli* | ISO TS 16649-3 | Live bivalve molluscs and live echinoderms, tunicates and gastropods | <230 MPN / 100g of flesh and intravalvular liquid[2] |

Adapted from EC (2007). [1]Absence in 25 g before the food has left the immediate control of the food business operator who has produced it. [2]A pooled sample comprising a minimum of 10 individual animals. RTE = Ready to eat; CFU = colony forming units; CRL= Central Reference Laboratory; MPN = most probable number.

## 1.2 Diagnostic testing for foodborne pathogenic bacteria

The tests involved in the microbiological food examination include the qualitative detection of specific pathogens (presence or absence testing); enumeration of the total number of microorganisms and specific microorganisms, identification of special indicative groups or specific organisms and characterisation of isolated microorganisms. The methodology for the microbiological examination of food in the majority of analyses relies on conventional microbiological culture methods, although other methods are available. Using culture methods bacteria are identified based on phenotypic characteristics typical to the organism or common to a group of organisms.

An overview of a typical algorithm for the microbiological examination of food and the approximate times of each analysis is presented in Figure 1.1. In most cases, a 25g portion of the food sample must be tested as required by the Microbiological Criteria (EC, 2007). The first step in processing a food sample is that it is weighed, diluted ten-fold in a suitable diluent and then homogenised to separate the pathogen from the food matrix (Roberts *et al.,* 1995). The homogenate can subsequently be analysed for 'detection' of the pathogen - i.e. when the criteria require the level of acceptable organisms is 'absence' and/or enumeration of a target pathogen or indicator organism (Roberts *et al.,* 1995).

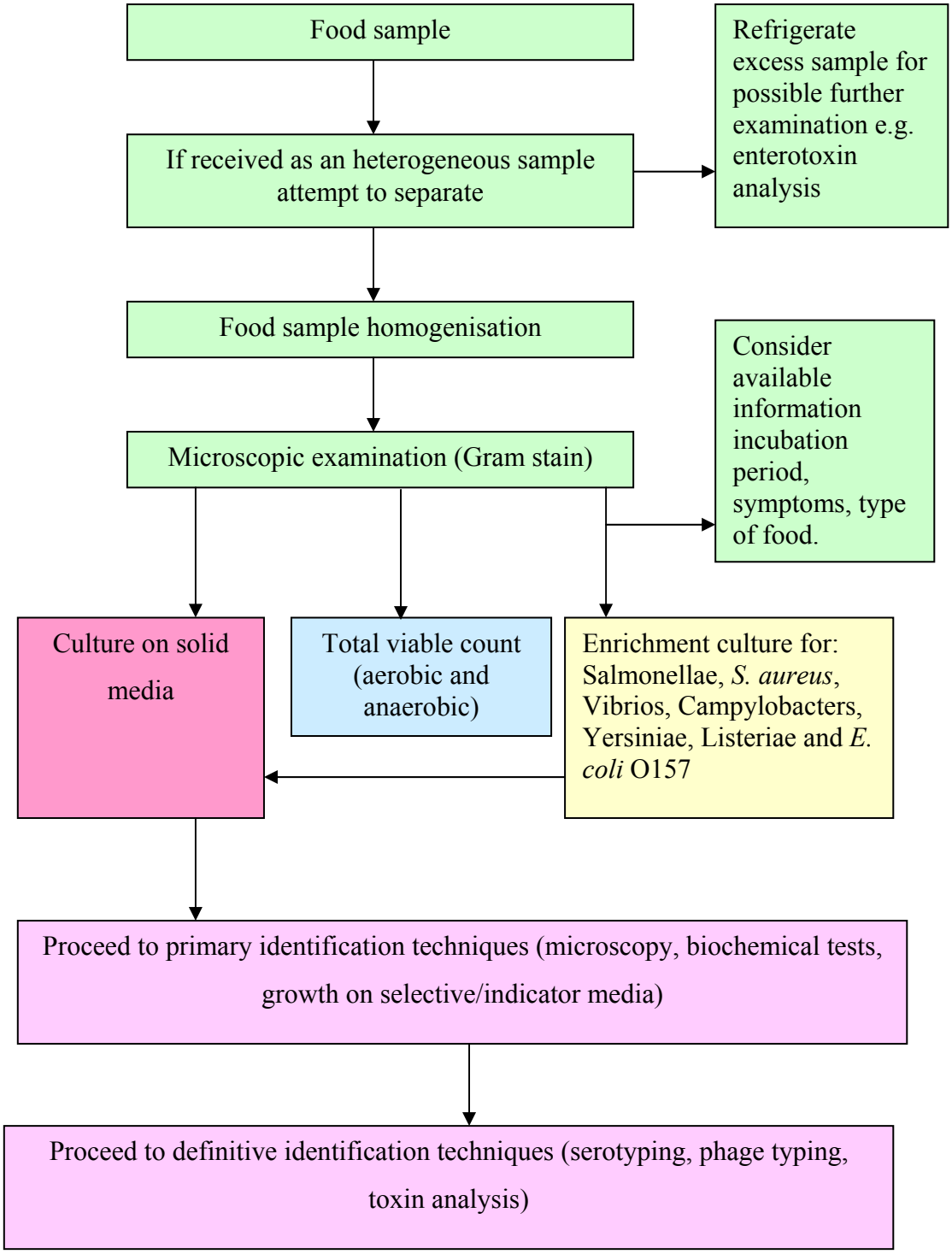



**Figure 1.1 Algorithm for the examination of food samples from food poisoning outbreaks**.

Adapted from Roberts *et al.* (1995)

### 1.2.1 **Microbiological analysis of food to the Microbiological Criteria**

The European Commission (EC) Microbiological Criteria stipulate the analytical method that must be used to measure each criterion (EC, 2007). These are International Organization for Standardization (ISO) methods, except for measurement of staphylococcal enterotoxins in milk samples. Other analytical methods can only be used if they are shown to give equivalent results and comply with the ISO protocol for validation of an alternative method (16140:2000). Of particular importance in the food safety criteria are the ISO methods used for the detection of *Salmonella* and *Listeria monocytogenes* in foods as they are applicable to a wide spectrum of foods. These bacterial pathogens are detected in foods by selective culture and subsequent identification of phenotypic characteristics (Roberts *et al.,* 1995). An example, of the detection of *Salmonella* spp. using the 'gold standard' culture technique described in the ISO 6579 method (ISO, 2002) follows.

Detecting *Salmonella* spp. in food requires sample dilution, ten-fold in Buffered Peptone Water and incubation for 24 hours. These culture conditions enable the resuscitation of cells that may have been sub-lethally damaged during the processes of food preservation. Following incubation, the resuscitation culture is sub-cultured in selective broths for a further 24 or 48 hours. These culture media contain agents that encourage the growth of the target organism rather than other microflora that may be present in the food sample. Without the pre-enrichment culture, the growth of the target organism could be inhibited in the selective broths. The selective broths described by the method for *Salmonella* are Rappaport-Vassiliadis Soya peptone broth (RVS) and Muller-Kauffmann Tetrathionate broth (MKTTn). RVS contains the selective agent malachite green whilst MKTTn contains bile salts and the antibiotic novobiocin for the specific culture of *Salmonella* spp.

Following the second incubation, the enrichment culture is sub-cultured on to solid media to allow the formation of characteristic colonies. Following

incubation cultured colonies give the typical appearance of the target pathogen, for example, *Salmonella* cultures on Xylose Lysine Decarboxlase Agar (XLD), will appear red with black centres as they generally ferment xylose, decarboxylate lysine and produce hydrogen sulphide. Culture-based methods then require up to five characteristic colonies to be tested using further biochemical methods to detect for phenotypic characteristics specific for the organism. Strains of *Salmonella* are tested for the production of acid, gas and hydrogen sulphide when grown on Triple Sugar Iron Agar, and the lack of urease enzyme when grown on Urease agar slopes. Strains of *Salmonella* are subsequently serotyped by performing agglutination with 'O' and 'H' antisera and may be phage typed for further strain discrimination.

### 1.2.2 **Improvements to culture detection techniques**

The detection of enteric pathogens has previously relied on culture-based techniques to isolate bacteria. As described above selective and non-selective media have been developed over many years to enhance recovery of pathogens. Automation of conventional microbiological techniques has resulted in an increased throughput of samples and decreased labour inputs, but has not reduced the overall time to produce results with these methods. Semi- or fully automated dilution procedures reduce the laboratory work by automatically aliquoting diluent (de Boer and Beumer, 1999). Equipment that reduces the laboratory work required for the enumeration of bacteria include the spiral plater, which automatically dispenses liquid samples to provide a dilution series of the culture on a single agar plate, and automated colony counters (de Boer and Beumer, 1999). However, complete automation in detection methods is highly desirable, but is not achievable with traditional methods (Bhunia, 2008).

Indicator agars have been developed for the primary identification of bacteria. For example, chromogenic and fluorogenic substrates have been used in media formulations as indicators of phenotypic characteristics. An example is a

chromogenic agar formulation ALOA (Agar *Listeria* Ottaviani and Agosti), which has replaced Oxford formulation for the detection and enumeration of *Listeria* spp. in the ISO 11290 part 2 method (ISO, 2004). In the ALOA medium, the chromogenic compound X-glucoside is added as substrate for the detection of β-glucosidase, which is common for all *Listeria* species. ALOA also has the advantages of enabling *L. monocytogenes* and *L. ivanovii* to be distinguished from other *Listeria* spp. The differentiation is based on the production of a phosphatidylinositol-specific phospholipase C in *L. monocytogenes* and *L. ivanovii* strains. The enzyme is used to hydrolyse the specific substrate added to the medium, resulting in an opaque clear-cut halo surrounding the *L. monocytogenes* colonies (Vlaemynck *et al.,* 2000). The medium can lead to the recognition of pathogenic *Listeria* in food samples up to three days earlier than traditional methods (Willis *et al.,* 2006).

Miniaturized identification systems that use biochemical and morphological tests are available for the characterisation of bacteria. An example, of a biochemical system of identification is the API® strips (Bio Merieux). These kits contain defined reactions for the identification of a group of organisms. Kits have the advantages of being standardised, pre-prepared and contain most of the tests required for the identification of bacterial strains. Many kits were validated with clinical isolates and so food isolates may not be identified if they have adapted to the environment of the food matrix.

Culture-based detection of bacterial pathogens in food takes anything from several days to weeks (Lauri and Mariani, 2009). Despite the implementation of automated techniques, the majority of the conventional culture methods still do not return results in a timely manner to allow real-time monitoring of food products at either their point of sale or when they have been implicated in food poisoning incidents. Faster and simpler methods would be a great advantage for many diagnostic purposes (Lauri and Mariani, 2009). Rapid methods have been considered to establish the microbiological quality of food. These

methods considerably reduce the reporting time compared to that of conventional culture based approaches (Abubakar *et al.,* 2007).

### 1.2.3 **Rapid methods for detecting bacterial pathogens in foods**

Possibly one of the most rapid methods currently being used in the food manufacturing industry is the measurement of Adenosine Triphosphate (ATP) bioluminescence for monitoring the hygiene of surfaces, producing a result in less than a minute. The method gives an estimation of total surface cleanliness including the presence of organic debris and microbial contamination (Aycicek *et al.,* 2006). The method employs luciferase which uses ATP and luciferin to produce bioluminescence detection of the light with a photomultiplier and gives a sensitive measurement of ATP concentration (Chittock *et al.,* 1998). Although it is an advantage that results are obtained in real-time in the field, this technology is a method of hygiene monitoring and does not identify the presence of specific organisms.

Traditionally immunoassays, which use the highly specific binding of antibody to antigen to identify bacteria or toxin, have been the most widely used of rapid methods for detection of pathogens in food samples. Of the immunoassays, enzyme-linked immunosorbent assay (ELISA) is the most common format of immunological methods available. Thompson *et al.* (2007) compared three commercially available ELISAs with selective plating and immunomagnetic separation (IMS) to detect *E. coli* O157:H7 on food samples including ground beef. The ELISA methods were found to be reliable and applicable for detection of *E. coli* O157:H7 in ground beef and there was a high-level agreement of detection between the ELISAs and IMS. Although ELISA methods can increase the speed of detection, they can be very labour intensive and so unless the methods are automated, they may become redundant in favour of large-scale testing methods (Abubakar *et al.,* 2007). New technologies, for example, antibody microarrays that are available in 96-mutliwell automated formats offer an approach for high-throughput screening

of large numbers of food samples for multiple pathogens and toxins (Gehring *et al.,* 2008).

Molecular methods detect nucleic acid, either DNA or RNA, as the target for identification analysis. All biochemical, immunological and other characteristics used for the detection of microorganisms are encoded in these molecules within an organism's genome or automously replicating plasmids. The genome of microorganisms may contain syntenous regions of DNA that are homologous across all members of a family or genus. However, there are also sequences unique to species, which can be used to identify specific microorganisms below the species level.

Genetic-based assays have the advantage of specific detection of an organism by targeting its unique nucleic acid. Methods utilizing nucleic acid targets include probe hybridisation and sequence amplification and detection techniques. Probe hybridisation involves the specific binding of a single-stranded oligonucleotide probe to a complementary DNA or RNA sequence in the target organism(s) of interest and direct detection of the target of interest for example, with digoxygenin, biotin or fluorescent dyes. Hybridisation assays are advantageous because of their rapidity results can be obtained in ninety minutes and probes can offer excellent specificity. DNA probes generally target ribosomal RNA (rRNA), taking advantage of the higher copy number of bacterial rRNA present in genomes and thus provide a naturally amplified target and affording a greater sensitivity. The disadvantages of probe hybridisation assays lie in their lack of sensitivity as at least $10^5$ to $10^6$ CFU $ml^{-1}$ of the bacteria are required for detection (Chan *et al.,* 1990; Hill and Keasler, 1991; Mabilat *et al.,* 1996). To overcome this lack of sensitivity, hybridisation is often used in combination with nucleic acid amplification techniques, such as the polymerase chain reaction (PCR), which has resulted in very specific and sensitive detection systems.

Currently the most sensitive molecular techniques involve an enzymatic amplification of a specific target nucleic acid before detection of the amplicon. *In vitro* nucleic acid amplification methods are capable of amplifying a single specific DNA or RNA sequence a million-fold in a few hours and provide a theoretical detection limit of one single bacterial cell (Erlich *et al.,* 1991). Common methods of enzymatic amplification methods are strand displacement amplification (SDA), Q-β replicase amplification, nucleic acid sequence-based amplification (NASBA), ligase chain reaction and branched DNA amplification technology. For example, NASBA was applied to detect *Escherichia coli* in water samples targeting a fragment of the clpB-mRNA and was shown to be specific and sensitive with a detection limit of one viable *E. coli* in 100ml and could be performed within 3-4h (Heijnen and Medema, 2009). At present the polymerase chain reaction (PCR) is the most versatile and widely adopted amplification technique (Abubakar *et al.,* 2007). PCR is described in section 1.2.4.

### 1.2.4 **Polymerase chain reaction**

The polymerase chain reaction (PCR) is the enzymatic amplification of a specific DNA target sequence using a DNA dependent DNA polymerase, which was developed by Kary Mullis in 1983 (Mullis, 1990). The PCR consists of three steps: (i) denaturation of double stranded target DNA; (ii) annealing of short DNA complementary DNA fragments (oligonucleotides) to single stranded DNA; and (iii) extension of the oligonucleotides by the DNA dependent DNA polymerase. These three steps are cyclically repeated during which both the original target and the amplification product (amplicons) are amplified. This leads to an exponential increase in the number of copies of the original DNA target resulting in amplification of one billion fold or greater depending on the number of PCR cycles performed.

### 1.2.5 **Application of PCR for the detection of pathogens**

PCR has been identified as a highly sensitive specific and rapid method for the identification of bacteria in pure cultures (Bej *et al.,* 1990). This versatility has lead to the application of this technology for the detection of bacterial pathogens in food. Many pathogen-specific PCR assays have been designed for the detection of pathogenic and spoilage microorganisms in food, for example, Hill and Kesler (1991) reviewed over eighty such PCR assays.

PCR test are attractive analyses due to their relative ease of use, low cost and potential application to large scale screening programs by means of automated technologies (Abubakar *et al.,* 2007). The use of PCR in clinical laboratories has vastly improved the detection of a wide range of pathogenic microorganisms (Espy *et al.,* 2006). Since its inception, PCR has been developed for use in many formats, including multiplexed reactions, nested reactions, and the inclusion of an initial reverse transcription phase for the application of PCR to RNA targets. More recent technology innovations have led to the development of real-time PCR systems, which are not only rapid but also enable detection of specific products in real-time (Holland *et al.,* 1991).

### 1.2.6 **Real-time PCR**

Real-time PCR combines PCR chemistry with fluorescent probe detection of amplified product in the same reaction vessel (Espy *et al.,* 2006). Real-time PCR instrumentation requires considerably less hands-on time and testing is simpler to perform than conventional PCR methods accelerated PCR thermocycling and detection of amplified product permits the provision of a test result much sooner for real-time PCR than for conventional PCR (Espy *et al.,* 2006).

The amplification of target is detected via a signal created when fluorescent molecules are bound to amplicons. The chemistry can be either sequence

independent or sequence-specific. The simplest and most cost effective methods employed are sequence independent DNA-binding dyes such as SYBR Green I and SYBR Gold, which fluoresce when bound to dsDNA (Karlsen *et al.,* 1995). The detection of real-time amplicons using sequence-specific fluorescently labelled oligonucleotides or probes gives the advantage of increased specificity over sequence independent methods. Several different sequence-specific probe formats exist, including hybridisation probes used in the Roche Light Cycler system (Wittwer *et al.,* 1997), hydrolysis probes used in Applied Biosystems TaqMan system (Heid *et al.,* 1996), scorpions (Whitcombe *et al.,* 1999) and molecular beacons (Tyagi and Kramer, 1996) as shown in Figure 1.2. Applied Biosystems TaqMan probes utilise the inherent 5' to 3' exonuclease activity of *Taq* DNA polymerase to simultaneously amplify and generate target specific signal (Holland *et al.,* 1991).

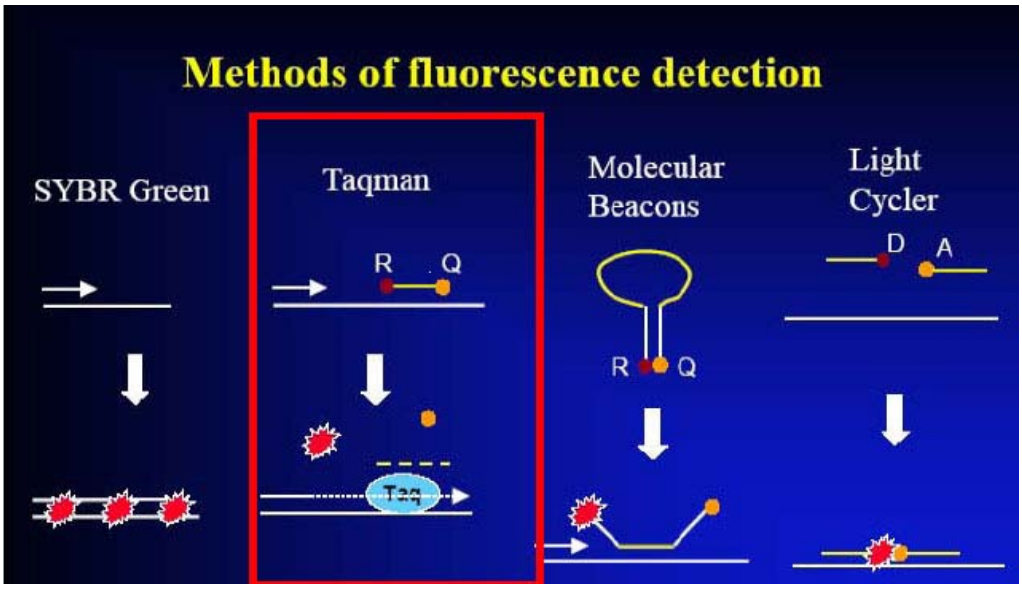



**Figure 1.2 Fluorescent detection methods employed for amplicon detection in real-time PCR assay.**

Taken from Gallup and Ackermann, (2006) TaqMan detection system is outlined in red box. R = reported, Q = quencher, D = donor, A = acceptor.

The combination of excellent sensitivity and specificity, low contamination risk, ease of performance and speed, has made real-time PCR technology an appealing alternative to conventional culture-based or immunoassay-based

testing methods used in the clinical microbiology for diagnosing many infectious diseases (Espy *et al.,* 2006). A further advantage of real-time PCR is the ability to use this technique to quantify the amount of target nucleic acid (Mackay *et al.,* 2002).

### 1.2.7 **Amplification of multiple gene targets simultaneously**

Multiplex PCR allows the simultaneous amplification of more than one target sequence in a single reaction by using multiple sets of oligonucleotides to amplify two or more targets of interest (Chamberlain *et al.,* 1988). For example, a multiplex real-time PCR using TaqMan probes have been reported for several foodborne pathogens, for example, Elizaquivel *et al.* (2008) described the simultaneous detection of *E. coli* O157:H7, *Salmonella* spp. and *S. aureus*. The assay had a sensitivity of $10^3$ $CFUg^{-1}$ for each pathogen when applied to artificially contaminated lettuce. Advantages of multiplex PCR include multiple targets that are amplified without significantly extra time, cost or sample volume. These assays can also include co-amplification of targets to act as controls in the reaction. The disadvantage of multiplex PCR is competition between oligonucleotide pairs can cause loss of PCR sensitivity (Klerks *et al.,* 2004).

### 1.2.8 **Nested PCR assays**

The use of nested PCR has been used to increase the sensitivity of PCR reactions. Nested PCR refers to a pair of PCRs run in series. The first PCR amplifies a sequence and in a second reaction, the (nested) oligonucleotides are complementary to targets within the first PCR product. Nested PCR assays increase sensitivity and yield a detectable product in cases where simple PCR fails to do so (Mullis and Faloona, 1987). This technique has a greater specificity because it uses four specific oligonucleotides, rather than the two used in conventional PCR. The detection of *Salmonella* by PCR was increased 1000-fold by applying a nested oligonucleotide stage to the reaction, resulting

in an assay with a sensitivity of one cell per ml of artificially contaminated water without the use of an enrichment step (Riyaz-Ul-Hassan *et al.,* 2004).

## 1.3 Challenges associated with the application of real-time PCR assays to detect foodborne bacteria

Despite the availability of PCR assays for many foodborne bacteria they have had a more limited application for testing food (McKillip and Drake, 2004). A major reason PCR has not been widely accepted for food hygiene testing for bacterial pathogens due to false-positive or false-negative PCR results when compared to the gold standard culture method of analysis. False positive PCR results are detected due to cross-contamination of the PCR, lack of specificity of the PCR target and the inability to distinguish between signals originating from viable cells and DNA released from dead cells. Reasons for false negative PCR results include the failure to detect pathogens due to inhibition of the DNA extraction or PCR, presence of a low level of contamination for example, one to 10 colony forming units, within a relatively large volume of food.

False-positive and false-negative PCR results from direct analysis of food samples have lead difficulties in equating these results to the microbiological safety of the food. Due to challenges posed to PCR analysis of food samples the robustness and suitability of PCR for detection of foodborne pathogens directly in food samples has been raised.

### 1.3.1 Reduction of potential false positive results due to cross contamination of the PCR

The exquisite sensitivity of the PCR means DNA contamination can result in false positive results (Kwok and Higuchi, 1989). Potential DNA cross-contamination has been minimised in many real-time PCR assays by incorporation of 2'-deoxyuridine 5'-triphosphate (dUTP), as a replacement for

2'-deoxythymidine 5'-triphosphate (dTTP) in the reaction, which producing PCR amplicons with an altered chemical structure (Sobek *et al.,* 1996). Then prior to amplification, a short incubation of the PCR reaction with 5' uracil n-glycosylase results in the degradation of DNA with incorporated dUTP, so removing cross contaminating amplicons carried over from previous reactions (Sobek *et al.,* 1996). Real-time PCR methods are within a closed system, which means they detect amplicons within the same instrument as the amplification. The closed system has the advantages of rapidity and as the amplification vessel is not opened after amplification the chance of cross-contamination is reduced.

### 1.3.2 **Approaches for the detection of viable cells in food sample testing**

A major challenge for applying PCR for diagnostics in food microbiology is the detection of DNA from dead cells, which can potentially result in false positives, where microbiologically safe food is interpreted as unsatisfactory. This is a particular problem in foods that have been treated to reduce or kill microorganisms, for example, dairy products that have been pasteurised may contain pathogen DNA but not viable organisms associated with foodborne illness (Rudi *et al.,* 2005a). Methods of decontaminating food often rely on inactivating pathogen cells rather than removing the organisms altogether, therefore the detection of DNA will give no indication of the effectiveness of a decontamination procedure (Malorny *et al.,* 2003b).

A viable microorganism can be defined as one that is capable of dividing and forming at least one live daughter cell when it is placed in a favourable environment (Roszak and Colwell, 1987). Microbiological detection of food pathogens generally includes incubation of the food in enrichment broth that allows the growth of viable organisms and the resuscitation of damaged organisms that can then be identified (Koo and Jaykus, 2000). The disadvantage of conventional culture methods is that they can take up to

several weeks to produce a confirmed result. A range of alternative approaches is available to assess bacteria viability as shown in Figure 1.3.

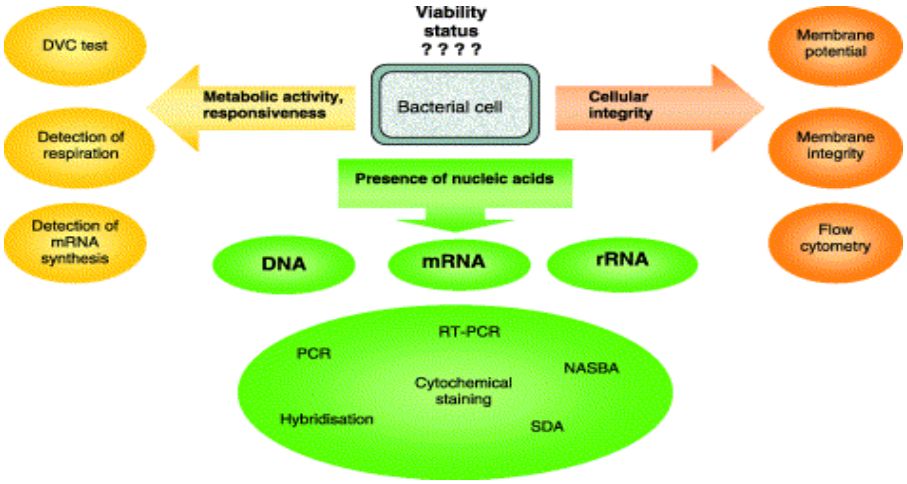



**Figure 1.3 Approaches that can be used for the assessment of bacteria viability.**

DVC = direct viable count; DNA = deoxyribonucleic acid; mRNA = messenger ribonucleic acid; rRNA = ribosomal ribonucleic acid; RT- PCR = reverse transcriptase PCR; PCR = polymerase chain reaction; SDA = strand displacement amplification.

Taken from Keer and Birch (2003).

It is possible to amplify DNA by PCR from both viable and non-viable cells and so it is possible that non-viable cells may be detected (Klein and Juneja, 1997). DNA has been demonstrated to persist in a PCR-detectable form in culture-negative environmental (Deere *et al.,* 1996) and clinical samples (Hellyer *et al.,* 1999). Detection of DNA rather than viable cells can therefore lead to the reporting of false-positive results. Detection of viable cells can be achieved by a cultural enrichment step prior to PCR, where viable bacteria increase in number sufficiently to surpass any residual non-viable organisms. However, culture enrichment techniques generally take a minimum of eighteen hours, which prolongs the analysis time (Klein and Juneja, 1997).

Since bacterial DNA can persist in the absence of viable infectious organisms, it is not a reliable marker of cell viability. As an alternative, ribosomal RNA (rRNA) has been investigated (McKillip *et al.,* 1999; Villarino *et al.,* 2000). The disadvantages of this approach include a long half-life of rRNA species and variable retention in the cell following a variety of bacterial stress treatments (Tolker-Nielsen *et al.,* 1997). Therefore, rRNA is considered a less accurate indicator of viability than messenger RNA (mRNA) (Bej *et al.,* 1991). The mRNA molecule is an intermediary in protein synthesis, and thus is only found in viable cells, it is also a highly labile with a very short half-life (minutes to seconds) and may provide a better indication of viability than a DNA or rRNA target for the detection of bacterial pathogens in food samples (Bej *et al.,* 1991).

### 1.3.3 **Choice of gene targets for PCR assays for specific detection of foodborne pathogens**

The genome of a microorganism contains homologous DNA sequences common to all members of a genus. However, there are also sequences that are less conserved and are unique to a species, sub-species or isolate, which can be exploited for identification or typing assays. The choice of sequence target in PCR identification is important for the specificity of the assay. A sequence that is specifically found in the bacterial species or group of interest but not present in other species or members of the group is necessary when interpreting a positive amplification and presence of a specific pathogen. Virulence genes are commonly used to identify pathogenic microorganisms for example, the haemolysin gene (*hly*) for *L. monocytogenes* (Nogva *et al.,* 2000) and enterotoxin gene for *C. perfringens* (*cpe*) (Amar *et al.,* 2005). The gene coding for 16S rDNA is found in all bacterial species and so has been exploited as a target for PCR amplification and used for identifying multiple pathogens in clinical and food samples. For example, Yang *et al.* (2002) described a novel real-time PCR to identify bacterial pathogens based on the 16S rDNA gene.

Multiplex PCR assays have been designed for the detection of bacteria in CSF using 16S and 23S rDNA genes (Greisen *et al.,* 1994; Kotilainen *et al.,* 1998).

For the microbiological analysis of food samples, the food is often screened for several types of bacterial pathogens and hygiene indicators simultaneously. This screening procedure can provide the analyst with a timely indication of the microbiological hygiene status of the sample. Multiplex PCR can be designed to target a gene common to all bacteria such as 16S rDNA, 23S rDNA or the 16S-23S rDNA spacer region, however the gene must contain conserved regions and have sufficient sequence diversity to allow species identification (Hong *et al.,* 2004).

The most important aspect of developing new PCR assays is the selection of gene target and sequence to ensure the exclusive detection of all target organisms (Abubakar *et al.,* 2007). PCR assays targeting genes coding for virulence factors can offer sequences homologous to the pathogen and can indicate potential pathogenicity. For example, the haemolysin gene (*hlyA*) was selected for the PCR detection of *L. monocytogenes* in this study. Haemolysin is used by *L. monocytogenes* to escape from destruction inside phagosomes, which is required for intracellular bacterial proliferation critical to the survival of a parasite within the cells of the eukaryote host (Vazquez-Boland *et al.,* 2001).

### 1.3.4 **Causes of false negative amplification due to inhibition to PCR from food samples**

When applied to food samples, the PCR can be inhibited or its sensitivity reduced severely (Rossen *et al.,* 1992). The reliability of PCR amplification methods depends in part on the presence of sufficient numbers of target molecules and the purity of the target template (Lampel *et al.,* 2000). Food components such as protein, fat, calcium ions have been shown to inhibit both nucleic acid extraction and amplification. The inhibitors generally act at one or

more of three essential points in the reaction, interference with cell lysis necessary for extraction of DNA, interference by nucleic acid degradation or capture, and inhibition of polymerase activity for amplification of target DNA (Wilson, 1997).

To reduce inhibition of PCR assays by food components, there are several methods that can be applied, including: food sample pre-treatment; DNA purification procedures; the use of DNA polymerases that are less sensitive to inhibitors, the use of amplification facilitators and dilution of the sample (Abu Al-Soud and Radstrom, 1989; Gregory *et al.,* 2006; Hoorfar *et al.,* 2004a). Bovine serum albumin has been used as a facilitator to reduce PCR inhibition in faecal and environmental samples (Kreader, 1996). The use of amplification facilitators or specialised DNA polymerases is dependent on the food type and the inhibitors associated with that food.

### 1.3.5 **Reduction of PCR inhibition using bacterial DNA preparation methods**

PCR amplification of DNA first requires the preparation of a nucleic acid sample that does not contain components that will inhibit the reaction and that contains a sufficient number of target molecules (Rantakokko-Jalava and Jalava, 2002). The composition of a food matrix can directly and significantly affect the PCR sensitivity with each food presenting its own set of challenges according to its composition and as a consequence no universal DNA extraction procedure exists (McKillip and Drake, 2004).

### 1.3.6 **Controls used to monitor PCR inhibition**

It is essential to demonstrate the validity of the PCR results through the use of appropriate controls as a failure to do so can lead to the reporting of both false negative and false positive results (Hoorfar *et al.,* 2004a). It is critical that

internal and external controls are included with each assay to monitor assay performance. External controls frequently comprise of the same reagents as are used in the detection assay, but instead of a test sample, they contain either known positive nucleic acid that should always give a PCR product, or no nucleic acid template providing a no-amplification control. External controls monitor the use of instrumentation, the assay reagents, and ensure that no amplification of cross-contaminating DNA has occurred.

Robustness of the PCR can monitored by the use of positive and negative test control reactions and the presence of PCR inhibitors should be monitored by the use of an internal amplification control (Malorny *et al.,* 2003b). When detecting pathogens in food, there is a strong requirement for the provision of controls that adequately assure the quality of all assays and processes. It is particularly important that the inhibition of PCR is controlled for in order to prevent the reporting of false negative results (Nogva *et al.,* 2000).

False-negative PCR results caused by inhibition of the amplification can be controlled by using an internal amplification or internal positive control. An internal control is, in contrast to an external control, amplified in the same tube as the target and is used to monitor the efficiency of each reaction (Lambertz *et al.,* 1998) providing assurance that amplification and detection are working effectively, with insufficient PCR inhibition to adversely affect the final result (Hoorfar *et al.,* 2004a; Rosenstraus *et al.,* 1998). An internal positive control is required in the reaction mix for a PCR method to comply with the International Standards Organisation guidelines for PCR testing of foodborne-pathogens in food samples.

Internal controls that are designed to be co-amplified with target specific oligonucleotides, but have an altered probe target site i.e. small deletion, an insertion or mutation are considered to be most accurate for detecting inhibition. In co-amplified internal controls the two amplicons are then differentiated by size or by the use of heterologous probes (Abdulmawjood *et*

*al.,* 2002; Cubero *et al.,* 2002; Malorny *et al.,* 2003a; Sachadyn and Kur, 1998; van der Zee *et al.,* 1993). However, if the same oligonucleotides are used to amplify both the control and the target amplicons, the overall detection limit of the assay may be reduced, especially if the target microbe is present at low levels and the internal control is assay specific. In previously published approaches for internal positive process controls, genes endogenous to the sample, for example, human mitochondrial cytochrome oxidase gene present in respiratory specimens (Fry *et al.,* 2004) or housekeeping genes i.e. genes required for basic functions of a cell, have been chosen.

Implementing control strategies using both internal and external controls provides quality assurance and increases the reliable application of PCR assays for the detection of bacterial DNA in heterogeneous food samples. Currently there is a lack of validated control material for monitoring performance of real-time PCR based assays for detecting bacterial pathogens in food samples. Control strategies to monitor the efficiency of DNA extraction and real-time PCR would be advantageous for analysing food samples. If introduced into an unprocessed food sample, an internal control can serve to monitor the complete nucleic acid extraction, amplification and detection procedure (Rosenstraus *et al.,* 1998). Given the control is used to monitor nucleic acid extraction from bacterial cells then it should be contained within a bacterial cell to give a true measure of extraction efficiency.

### 1.4 Overview and aim of the study

The cases of food related illness are reducing in the UK, but food-borne illness is still a significant problem due to gross underestimation of the total numbers of cases (Adak *et al.,* 2002). The detection of pathogenic microorganisms in foods is essential to ensure the basic requirement of a supply of food fit for human consumption. Conventional culture techniques currently used to detect pathogenic bacteria in food samples are time consuming and labour intensive, and so increasing seen as unfit to meet the requirements of a modern food microbiology laboratory. Advances in nucleic acid detection technologies have seen a plethora of real-time PCR-based methods for the detection of a wide range of microbial pathogens (Espy *et al.,* 2006; McKillip and Drake, 2004).

From a public health perspective, faster detection times are essential to prevent the spread of infectious disease or the identification of a continuing source of infection (Abubakar *et al.,* 2007). However, the food industry and food microbiology laboratories have been slower to adopt molecular methods because of the problems associated false positive and false negative PCR results. For PCR to become a standardised robust method that is employed in commercial and public health food hygiene testing the PCR assays must be robust and equivalent the results obtained by culture based methods.

### 1.4.1 **Key issues identified for the application of PCR for the detection of bacterial pathogens directly in food samples**

From the literature review, the following key issues were identified:

- PCR is a rapid, sensitive, specific, high thought-put method of detecting pathogenic bacteria; however, it is not reliable when applied to detect contaminating bacteria in food samples.

- PCR methods have resulted in false-positive and false-negative results when compared to the gold standard of culture based analysis.

- False positive PCR result from: (a) lack of correlation between a positive PCR test and detection of viable pathogenic bacteria, and (b) cross contamination of this extremely sensitive reaction.

- False negative PCR results are due to a lack of sensitivity when applied to a large volume food samples that are contaminated with low levels of pathogenic bacteria and inhibition of the PCR or DNA extraction by food components.

- There is a lack of generic bacterial DNA extraction methods for application to food samples.

- There is a lack of adequate controls described in published PCR assays applied to detect bacterial pathogens in contaminated food samples.

- If PCR methods can be modified to be reliable then analysis of food for the presence pathogenic bacteria then this would provide a considerable improvement in through-put and time from analysis to results which could help in the provision of food that is fit to consume.

### 1.4.2 **Hypothesis and specific objectives of the study**

The overall hypothesis to be tested in this thesis is whether PCR assays can be developed that are equal or better, than cultural detection in terms of robustness and reliability when applied to the detection of bacterial pathogens directly in food samples.

The specific objectives of the work were to:

- Evaluate MagNA Pure™ automated extraction for preparing bacterial DNA from contaminated food samples for detection in PCR assays, because of the current lack a reliable generic method (Chapter 3);

- Develop an internal positive process control for the DNA extraction and PCR applicable for detecting bacterial pathogens in food samples because the false negatives resulting from inhibition must be identified to produce a robust assay (Chapter 4);

- Investigate a molecular method for the detection of viable *L. monocytogenes* cells to stop the detection of false positive PCR resulting from dead bacteria present in a sample and increasing the correlation of PCR assays with culture analysis (Chapter 5);

- Develop specific PCR assays for identification of *E. coli* O157:H7, *L. monocytogenes*, *S. aureus*, *S. enterica*, *C. perfringens*, *C. jejuni* and *C. coli* that can be multiplexed to enable a high through-put analysis of food samples because this will allow samples to be screened for the presences of multiple bacteria simultaneously giving a more rapid and comprehensive result (Chapter 6); and

- Apply optimised PCR assays for the detection of pathogens to naturally-contaminated samples in parallel with standard culture techniques and establish the reliability of the PCR assays by correlation between each type of analysis (Chapter 7).

# CHAPTER 2

MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Control bacterial strains

Control bacterial strains used in this work are listed in Table 2.1. The control strains were obtained from the National Collection of Type Cultures (NCTC) unless otherwise stated. The control strains: *L. monocytogenes* NCTC 12427, *C. jejuni* NCTC 11168, *C. perfringens* NCTC 8239 and *S. enterica* NCTC 13349 were selected as whole genome sequences have been published that enabled gene copy calculations.

*C. coli* NCTC 12110 was selected as a control strain as recommended by The Campylobacter and Helicobacter Reference Unit (CHRU), HPA. *E. coli* O157:H7 NCTC 12900 was selected as it is serotype O157:H7 but is non-toxigenic strain so can be handled as an ACDP category 2 organism. *S. aureus* NCTC 6571 was selected as it is the NCTC Food and dairy recommended control strain. Bacterial strains used in the construction of *E. coli* GFP are described in section 2.6.

**Table 2.1 Bacterial control strains obtained from National Collection of Type Cultures.**

| Microorganism | NCTC number |
|---|---|
| *Arcobacter butzleri* | 12481 |
| *Arcobacter skirrowii* | 12713 |
| *Bacillus cereus* | 7464 |
| *Bacillus subtilis* | 10400 |
| *Campylobacter coli* | 12110 |
| *Campylobacter concisus* | 11485 |
| *Campylobacter fetus* subsp. *fetus* | 10842 |
| *Campylobacter fetus* subsp. *venerealis* | 10354 |
| *Campylobacter jejuni* | 11168 |
| *Campylobacter lari* | 11352 |
| *Campylobacter mucosalis* | 11000 |
| *Campylobacter upsaliensis* | 11541 |
| *Clostridium perfringens* | 8239 |
| *Cronobacter sakazakii* | 9238 |
| *Escherichia coli* | 9001 |
| *Escherichia coli* O157:H7 | 12900 |
| *Helicobacter pylori* | 11637 |
| *Klebsiella aeruginosa* | 9528 |
| *Listeria grayi* | 10815 |
| *Listeria innocua* | 11288 |
| *Listeria ivanovii* | 11846 |
| *Listeria monocytogenes* | 11994 and 12427 |
| *Listeria seeligeri* | 11856 |
| *Listeria welshimeri* | 11857 |
| *Pseudomonas aeruginosa* | 10662 |
| *Salmonella bongori* | 12419 |
| *Salmonella enterica* serovar Enteritidis Phage type 4 | 13349 |
| *Salmonella enterica* serovar Nottingham | 7832 |
| *Staphylococcus aureus* | 1803 and 6571 |
| *Staphylococcus epidermidis* | 11047 |
| *Vibrio furnissii* | 11218 |
| *Vibrio parahaemolyticus* | 10885 |
| *Yersinia enterocolitica* | 10460 |

NCTC = National Collection of Type Cultures

### 2.1.2 **Wild-type bacterial strains and DNA from wild-type strains**

The wild-type bacterial strains used in this study are listed in Table 2.2. The Food Safety Microbiology Laboratory (FSML) or Laboratory of Enteric Pathogens at the HPA supplied the wild-type bacteria or DNA. DNA samples rather than isolates were supplied for the Advisory Committee on Dangerous Pathogens (ACDP) containment level three organisms: verocytotoxic *E. coli*,

*S. enterica* serovar Typhi and Paratyphi. Cultures or nucleic acid samples supplied were either phenotypically confirmed isolates or genotypically confirmed by the laboratories at the HPA.

**Table 2.2 Wild-type bacterial strains, viral and parasitic microorganisms used in this study.**

| Microorganism | Number of isolates |
|---|---|
| *Campylobacter coli* | 10 |
| *Campylobacter jejuni* | 10 |
| *Clostridium perfringens* | 10 |
| *Enterobacter* spp. | 2 |
| *Escherichia coli* | 10 |
| *Escherichia coli* O157:H7[1] | 50 |
| *Hafnia alvei* | 5 |
| *Listeria monocytogenes* | 10 |
| *Proteus sp.* | 1 |
| *Salmonella enterica*[1] | 86 |
| *Serratia liquefaciens* | 1 |
| *Staphylococcus aureus* | 105 |
| *Shigella boydii* | 5 |
| *Shigella dysenteriae* | 5 |
| *Shigella flexneri* | 5 |
| *Shigella sonnei* | 5 |
| *Yersinia rohdei* | 3 |
| *Yersinia mollaretii* | 1 |
| *Yersinia bercovieri* | 1 |
| *Yersinia intermedia* | 1 |
| *Yersinia frederiksenii* | 10 |

[1] DNA supplied for *E. coli* O157:H7, *S. enterica* serovar Typhi and Paratyphi

### 2.1.3 Parasitic and viral nucleic acid samples

Viral and parasitic nucleic acid used in this study is detailed in Table 2.3 was extracted from faecal samples using Boom method (section 2.5.5) and was kindly supplied by Dr. Corinne Amar (FSML, HPA).

**Table 2.3 Parasite and viral nucleic acid samples used in this study.**

| | Microorganism | Number of samples |
|---|---|---|
| Parasite | Cryptosporidium | 1 |
| Viral | norovirus | 1 |
| | rotavirus | 1 |
| | sapovirus | 1 |

### 2.1.4 **Food and environmental samples**

Soft cheese, coleslaw and semi-skimmed milk samples used for spiking experiments (Chapter 3) were purchased from local supermarkets and used before the manufacturer's "use by" date. Food and environmental samples used for the field trial (Chapter 7) were supplied as enrichment cultures from the Preston and London Food, Water and Environmental Laboratories. All food samples were stored at 4 $^{o}$C until used.

### 2.1.5 **Cerebrospinal fluid and serum samples.**

Cerebrospinal Fluid (CSF) and serum sample were received by The Food Safety Microbiological Laboratory (FSML) from HPA or clinical microbiology laboratories had been used for the detection of *L. monocytogenes* in the diagnosis of listeriosis. Serum and CSF samples were stored at 4 $^{o}$C until used.

## 2.2 General reagents and consumables

The following reagents were used throughout: DNase and RNase-free 1.5 ml tubes (Sarstedt, Leicester UK), nuclease-free water (Promega, Southampton UK) and ethanol (99.7-100 % [v:v]) (BDH, Lutterworth, UK). Ethanol was diluted to the appropriate concentrations (70 %, 50 % [v:v]) using nuclease-free water. The 1 x TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8; Sigma, UK) was prepared using nuclease-free water. The 1 x TAE buffer (Tris acetate 40 mM, EDTA 1 mM pH 8.3; Invitrogen, Paisley, UK) was prepared using distilled water.

### 2.2.1 Culture media used in this study

Solid and liquid culture media used in this study and their formulations are listed in Tables 2.4 and 2.5 respectively. All media (except Tripticase Soya Agar; Luria-Bertani Broth and Agar) were prepared in the HPA Media Production Department (HPA, London) from reagents supplied by Oxoid, Basingstoke, UK unless otherwise stated. The Luria-Bertani Broth and Agar were purchased as dehydrated media from Sigma and prepared following the manufacturer's instructions. Tripticase Soya Agar was purchased as dehydrated media from Oxoid and prepared according to the manufacturer's instructions.

**Table 2.4 Solid media used in this study.**

| Media | Abbreviation | Components per litre (Oxoid product code) |
|---|---|---|
| Baird-Parker | BPM | Tryptone 10.0g; 'Lab-Lemco' powder 5.0g; yeast extract 1.0g; $C_3H_3NaO_3$ 10.0g; glycine12.0g; LiCl 5.0g agar 20.0g; pH 6.8 ±0.2; (CM0275); tellurite (SR54). |
| Blood Agar | BA | 'Lab-Lemco' powder 10.0g; peptone neutralised 10.0g; NaCl 5.0g; agar 15.0g; pH 7.3 ±0.2; (CM55). Defibrinated Horse Blood (SR50). |
| Blood Agar with nutrient agar layer | BN | See Blood agar and nutrient agar formulations. The layered agar used 12 ml nutrient agar and 15 ml of horse Blood agar. |
| Columbia Blood Agar with peptone water layer | NEOH | Columbia Blood Agar base 39g (CM331) sterile horse blood; Neomycin sulphate (75 mg $ml^{-1}$, Sigma) 1.33 ml; pH 7.3 ±0.2. Peptone No.1 (Lab M, Bury, UK); NaCl 5.0g; Agar 15.0g pH 6.8 ±0.2. |
| Brilliant Green Agar (modified) | BGA | 'Lab-Lemco' powder 5.0g; peptone 10.0g; yeast extract 3.0g; $Na_2HPO_4$ 1.0g; $NaH_2PO_4$ 0.6g; lactose 10.0g; sucrose 10.0g; phenol red 0.09g; brilliant green 0.0047g; Agar 12.0g; pH 6.9 ± 0.2; (CM329) Sodium sulphacetamide 1000mg, sodium mandelate 250mg (SR87). |
| Campylobacter Blood –free selective Agar | CCDA | Nutrient Broth No.2 25.0g; bacteriological charcoal 4.0g casein hydrolysate 3.0g; sodium desoxycholate1.0g; $FeSO_4$ 0.25g; $C_3H_3NaO_3$ 0.25g; Agar 12.0g; pH 7.4 ±0.2; (CM739) Cefoperazone 32mg; Amphotericin B 10mg (SR155). |
| Listeria Selective Agar (oxford formulation) | Oxford | Columbia Blood Agar base 39.0g; aesculin 1.0g; ferric ammonium citrate 5g; LiCl 15.0g; pH 7.0 ± 0.2 (CM 856). Cycloheximide 200mg; colistin sulphate 10mg; acriflavine 2.5mg; cefotetan 1.0mg; fosfomycin 5.0mg (SR140). |
| Luria Agar | | Tryptone (pancreatic digest of casein) 10g, yeast extract 5g, NaCl 10g, Agar 15g. |
| MacConkey Agar | MAC | Peptone 20.0g; lactose 10.0g; bile salts 5.0g; NaCl 5.0g; neutral red 0.075g; Agar 12.0g; pH 7.4 ± 0.2 (CM7). |
| Cefixime-Tellurite MacConkey Agar | CT-SMAC | See MacConkey Agar above (CM7); plus potassium tellurite 2.5mg; cefixime 0.05mg (SR172). |
| Nutrient Agar | NA | 'Lab-Lemco' powder 1.0g; yeast extract 2.0g; peptone 5.0g; NaCl 5.0g; Agar 15.0g; pH 7.4 ± 0.2 (CM3). |
| Tryptose Sulphate Cycloserine Agar | TSC | Tryptose 15.0g; soya peptone 5.0g; yeast extract 5.0g; sodium metabisulphite 1.0g; ferric ammonium citrate 1.0g; Agar 19.0g; pH 7.6 ± 0.2. (CM0587); D-cycloserine 200mg (SR88). |
| Plate Count Agar | PCA | Tryptone 5.0g; yeast extract 2.5g; glucose 1.0g; Agar 9.0g; pH 7.0 ± 0.2 (CM325). |
| Triptone Soya Agar | TSA | Tryptone 15.0g; soya peptone 5.0g; NaCl 5.0g; Agar 15.0g; pH 7.3 ± 0.2 (CM0131). |
| Xylose Lysine Deoxycholate Agar | XLD | Yeast extract 3.0g; L-lysine HCI 5.0g; xylose 3.75g; sucrose 7.5g; sodium desoxycholate 1.0g; NaCl 5.0g; $Na_2S_2O_3$ 6.8g; ferric ammonium citrate 0.8g; phenol red 0.08g; Agar 12.5g; pH 7.4 ± 0.2 (CM0469). |

pH values at 25 $^{o}$ C.

**Table 2.5 Liquid media used in this study.**

| Media | Abbreviation | Components per litre (Oxoid product code) |
|---|---|---|
| Bolton Broth | BB | Meat peptone 10g; lactalbumin hydrolysate 5g; yeast extract 5g; NaCl 5g; alpha-ketoglutaric acid 1g; $C_3H_3NaO_3$ 0.5g; Sodium metabisulphite 0.5g; $Na_2CO_3$ 0.6g; haemin 10mg; pH 7.4 ± 0.2 (CM0983). Cefoperazone 20mg; Vanomycin 20mg; Trimethoprim 20mg; Cycloheximide 50mg; (SR0183). Lysed blood 50mL (SR0048). |
| Brain Heart Infusion Broth | BHI | Calf brain infusion solids 12.5g; beef heart infusion solids 5.0g; proteose peptone 10.0g; glucose 2.0g; NaCl 5.0g; $Na_2HPO_4$ 2.5g; pH 7.4 ± 0.2 (CM0225). |
| Buffered Peptone Water | BPW | Peptone 10.0g; NaCl 5.0g; $Na_2HPO_4$ 3.5g; Potassium dihydrogen phosphate 1.5g; pH 7.2 ± 0.2 (CM0509). |
| Cooked Meat Broth | CMB | Heart muscle 454.0g; Peptone 10.0g; 'Lab-Lemco' powder 10.0g; NaCl 5.0g; Glucose 2.0g; pH 7.2 ± 0.2 (CM0081). |
| Fraser Broth | FB | Proteose peptone 5.0g; Tryptone 5.0g; 'Lab-Lemco' powder 5.0g; Yeast extract 5.0g; NaCl 20.0g; $Na_2HPO_4$ 12.0g; Potassium dihydrogen phosphate 1.35g; Aesculin 1.0g; LiCl 3.0 g; pH 7.2 ± 0.2; (CM0895). Ferric ammonium citrate 0.5g; Nalidixic acid 20.0mg; Acriflavine hydrochloride 25mg; (SR0156). |
| Half Fraser Broth | HFB | See Fraser Broth above (CM0895) plus ferric ammonium citrate 0.5g; nalidixic acid 10.0mg; acriflavine hydrochloride 12.5mg; (SR0166). |
| Luria Broth | LB | Tryptone (pancreatic digest of casein) 10g, yeast extract 5g, NaCl 10g. |
| Maximum Recovery Diluent | MRD | Peptone 1.0g; NaCl 8.5g; pH 7.0 ± 0.2 (CM0733). |
| Muller-Kauffmann Tetrathionate Broth | MKTTn | Tryptone 7.0g; soya peptone 2.3g; NaCl 2.3g; $CaCO_3$ 25.0g; $Na_2S_2O_3$ 40.7g; ox bile 4.75g; (CM1048). Novobiocin 20mg (SR0181). |
| Nutrient Broth | NB | 'Lab-Lemco'powde 1.0g; yeast extract 2.0g; peptone 5.0g; NaCl 5.0g; pH 7.4 ± 0.2; (CM0001). |
| Pages Saline | | NaCl 0.12g; magnesium sulphate 0.004g; CaCl 0.004g; sodium phosphate 0.14g; $KH_2PO_4$ 0.136g; pH 6.8 ± 0.2. |
| Rappaport-Vassiliadis Soya Peptone Broth | RVS | Soya peptone 4.5g; NaCl 7.2g; potassium dihydrogen phosphate 1.26g; di-potassium hydrogen phosphate 0.18g; MgCl 13.58g; malachite green 0.036g; pH 5.2 ± 0.2 (CM0866). |
| Selenite Cystine Broth | SC | Tryptone 5.0g; lactose 4.0g; $Na_2HPO_4$ 10.0g; L-Cystine 0.01g; pH 7.0 ± 0.2 (CM0699). Sodium biselenite 4g (LP0121); pH 7.0 ± 0.2. |
| Super Optimal Catabolite Repression Medium | SOC | Purchased as a prepared liquid containing: Tryptone (pancreatic digest of casein, 2 % (w/v), yeast extract 0.5% (w/v); NaCl 8.6 mM, KCl 2.5 mM, $MgSO_4$ 20 mM, glucose 20 mM pH 7.0 ± 0.2. |

pH values at 25 $^{o}$ C.

### 2.2.2 **Oligonucleotides used for 5' nuclease real-time PCR, NASBA and Bio-plex assays**

All the oligonucleotides were purchased from Invitrogen, with the exception of P1, P1T7 and P2 that were purchased from Proligo (Paris, France). All of the oligonucleotide sequences are provided in Table 2.6. The oligonucleotide positions in their respective gene targets are shown in Figure 8.1 (Appendix 1). All oligonucleotides and probes were purified by high performance liquid chromatography by the manufacturer and stored in the dark. A second aliquot of the reverse oligonucleotides were labelled with biotin for the Bio-plex assays (oligonucleotides show in Table 2.6). Oligonucleotides were diluted to stock solutions (100 mM) and working solutions with nuclease-free water and were stored at – 20 $^{o}$C and + 4 $^{o}$C respectively until used.

## Table 2.6 PCR, NASBA and Bio-plex Oligonucleotide sequences.

| Micro-organism (gene) | Name | Sequence (5'-3') | Source |
|---|---|---|---|
| | | NASBA | |
| *L. monocytogenes* (*hlyA*) | P1 | GCG TAA GTC TCC GAG GTT G | This study |
| | P2 | ATC TCA AGT GTG GCG TAT GG | This study |
| | P1T7[1] | AATTCTAATACGACTCACATGGG GCG TAA GTC TCC GAG GTT G | This study |
| | P2T7[1] | AATTCTAATACGACTCACTATAGGGAT CTC AAG TGT GGC GTA TGG | This study |
| | NASBA oligo | GTT AGT TCT ACA TCA CCT GAG ACA GA | This study |
| | BTP1[1] | AATTCTAATACGACTCACTATAGGGAG ATA ACC TTT TCT TGG CGG CAC A | (Blais *et al.,* 1997) |
| | BTP2 | GTC CTA AGA CGC CAA TCG AA | (Blais *et al.,* 1997) |
| | | PCR | |
| *L. monocytogenes* (*hlyA*) | hlyOR | TTT CTT GGC GGC ACA TTT G | This study |
| | hlyOF | CAT GGC ACC ACC AGC ATC T | This study |
| | RT-hlyAR | ATT GTA ATC CGC GCC TTG TAT ATA | This study |
| | hlyAF | TGC AAG TCC TAA GAC GCC A | (Nogva *et al.,* 2000) |
| | hlyAR[2] | CAC TGC ATC TCC GTG GTA TAC TAA | (Nogva *et al.,* 2000) |
| | hlyARX | TTT CTT CGC GGC ACA TTT G | This study |
| *C. coli* (*ceuE*) | ceuEF | AAG CTC TTA TTG TTC TAA CCA ATT CTA ACA | (Best *et al.,* 2003) |
| | ceuER[2] | TCA TCC ACA GCA TTG ATT CCT AA | (Best *et al.,* 2003) |
| | ceuERX | GAA CGC GCA CAA GCA ATA C | This study |
| *C. perfringens* (*cpa*) | cpaF | CTA GAT ATG AAT GGC AAA GAG GAA ACT A | (Amar *et al.,* 2005) |
| | cpaR[2] | TTA GCA GGA TGA TAT GGA GTA TCT ATA TCTC | (Amar *et al.,* 2005) |
| | cpaRX | CGT TGA TAG CGC AGG ACA ATG | This study |
| *C. perfringens* (*cpe*) | cpeF | GGT AAT TTA TAT GAT TGG CGT TCT TCT | (Amar *et al.,* 2005) |
| | cpeR[2] | CTA AGA TTC TAT ATT TTT GTC CAG TAG CTG TAA | (Amar *et al.,* 2005) |
| | cpeRX | GTG AAA TTA GAA CAG TCC TTA AGG TGA TG | This study |
| *E. coli* O157:H7 (*eaeγ*) | eaeγF | ATT ATG CTG ATG CTA TGT CCA TTT G | This study |
| | eaeγR[2] | TGT TTA ATC CAA GCA GTT ATT GAG TTC | This study |
| | eaeγRX | GAC ATC TAG TGA GCA GCG TTC TG | This study |
| *E. coli* GFP and pAG408 (*gfp*) | gfpF | CCT GTC CTT TTA CCA GAC AAC CA | This study |
| | gfpR[2] | GGT CTC TCT TTT CGT TGG GAT CT | This study |
| | gfpRX | CAG CTG CTG GGA TTA CAC TG | This study |
| *S. enterica* (*iroB*) | iroBF | CCA AGA GAT CTG GCG TGG ATA G | This study |
| | iroBR[2] | GGA CGT ATT GCA TGG AGA TAA CC | This study |
| | iroBRX | GGG AAG AAT GGT GGG AAC GTA | This study |
| *C. jejuni* (*mapA*) | mapAF | CTG GTG GTT TTG AAG CAA AGA TT | (Best *et al.,* 2003) |
| | mapAR[2] | CAA TAC CAG TGT CTA AAG TGC GTT TAT | (Best *et al.,* 2003) |
| | mapARX | GTC AAC CAC AAC TAT TCC TCT AGC | This study |
| *S. aureus* (*nuc*) | nucF | ATT GAA GTC GAG TTT GA CAA AGG T | This study |
| | nucR[2] | CTT TAG TTC GTC CAA GGC TTG GC | This study |
| | nucRX | GTC GAT TCA GGT CAA TAA TGC TCA | This study |
| Eubacteria (16S rDNA) | uniF | CCA TGA AGT CGG AAT CGC TAG | (Corless *et al.,* 2000) |
| | uniR | ACT CCC ATG GTG TGA CGG | (Corless *et al.,* 2000) |

[1]The T7 RNA polymerase-binding sites are underlined.

[2]labelled with biotin for Bio-plex assays

### 2.2.3 **Probes used for 5' nuclease real-time PCR and NASBA assays**

The probe sequences for NASBA and PCR and are provided in Table 2.7, the probe positions in their respective gene targets are shown in Figures 8.1 and 8.2 respectively (Appendix 1). Real-time PCR probes were labelled at the 5' with the reporter dye FAM™ (carboxyfluorescein) and at the 3' with the quencher dye TAMRA (6-carboxy-*N*,*N*,*N*',*N*' tetramethylrhodamine) and HPLC purified. The real-time PCR probes were purchased from Eurogentec (Seraing Belgium). The molecular beacon probe for NASBA reactions was labelled at the 5' with FAM™ and the Black hole quencher 1 (BHQ1) – phosphoramidite was used as the quencher at the 3'. The beacon was purchased from Proligo. The probes were diluted in 1 x TE buffer (Sigma) and the stock and working solutions were stored at – 20 $^{o}$C and + 4 $^{o}$C respectively until used.

**Table 2.7 Probe sequences for 5' nuclease real-time PCR assays.**

| Micro-organism (gene) | Sequence (5'-3') | Source |
|---|---|---|
| | NASBA | |
| *L. monocytogenes* (*hlyA*) | CGC GAT CTC TGT CTC AGG TGA TGT AGA ACT AAC GAT CGC G | This study |
| | PCR | |
| *C. coli* (*ceuE*) | TTG GAC CTC AAT CTC GCT TTG GAA TCA TT | (Best *et al.,* 2003) |
| *C. perfringens* (*cpa*) | TTG AAT TCC AAC ATC GCT AAT GTA TAA AAG CCC TTT | (Amar *et al.,* 2005) |
| *C. perfringens* (*cpe*) | TTG GAC CTC AAT CTC GCT TTG GAA TCA TT | (Amar *et al.,* 2005) |
| *E. coli* O157:H7 (*eaeγ*) | ACC ATC CAC ACA GAC GGT ATT GTC AGA TAT TTA TGA | This study |
| *E. coli* GFP and pAG408 (*gfp*) | TAC CTG TCC ACA CAA TCT GCC CTT TCG | This study |
| *L. monocytogenes* (*hlyA*) | CGA TTT CAT CCG CGT GTT TCT TTT CG | (Nogva *et al.,* 2000) |
| *S. enterica* (*iroB*) | ACC GCC CAG CAT GAG CAT ACT GC | This study |
| *C. jejuni* (*mapA*) | CTC ATA CCC TTG GAC TCA AAA GCT TAA TTT ACA CTT AAC A | (Best *et al.,* 2003) |
| *S. aureus* (*nuc*) | CCA TCA GCA TAA ATA TAC GCT AAG CCA CGT CC | This study |
| Eubacteria (16S rDNA) | CGG TGA ATA CGT TCC CGG GCC TTG TAC | (Corless *et al.,* 2000) |

All probes were labelled with 5' reporter dye: FAM™ (carboxyfluorescein). The 3' quencher dye used was TAMRA (6-carboxy-*N*,*N*,*N*',*N*'-tetramethylrhodamine) except for the molecular beacon that was labelled with Black hole quencher 1 (phosphoramidite). The stem of the molecular beacon sequence is underlined.

### 2.2.4 **Probes used for Bio-plex assay**

The probes for the Bio-plex assay were purchased from Sigma Genosys and the sequences are given in Table 2.8. The probe positions in their respective gene targets are shown in Figure 8.2 (Appendix). Oligonucleotides used as capture probes in the Bio-plex assays were synthesized with an amino-modified 12-carbon spacer at the 5' end of each probe and HPLC purified.

**Table 2.8 Probe sequences for Bio-plex assay.**

| Microorganism | Gene target | Sequence 5' to 3' |
|---|---|---|
| *L. monocytogenes* | *hlyA* | AAG AAA CAC GCG GAT GAA AT |
| *C. perfringens* | *cpe* | CCT TGG ACT CAA AAG CTT AA |
| *C. perfringens* | *cpa* | CTA CAT TCT ATC TTG GAG AG |
| *C. jejuni* | *mapA* | GGA ATT CAA TGT TGT GCC AA |
| *C. coli* | *ceuE* | TCA ATC TCG CTT TGG AAT CA |
| *E. coli* O157:H7 | *eaey* | GAT ATT TAT GAC TCA TGG GG |
| *S. enterica* | *iroB* | CAG CAT GAG CAT ACT GCA AA |
| *S. aureus* | *nuc* | CGT GGC TTA GCG TAT ATT TA |
| *E. coli* GFP | *gfp* | TTA CCT GTC CAC ACA ATC TGC |

All Probes were labelled with 5' amine C12 group for attachment to the microspheres.

## 2.3 Instrumentation

A list of the instruments used and their suppliers for each process is detailed in Table 2.9.

**Table 2.9 Instruments and suppliers used for the processes in this study.**

| Process | Name of instrument | Supplier |
|---|---|---|
| General centrifugation | Micromax RF | Thermo Life Sciences, Basingstoke, UK |
| PCR, NASBA and real-time RT-PCR assays | ABI Prism 7000 or 7700 sequence detection system | Applied Biosystems Warrington, England |
| Conventional PCR | T3 Biometra Thermocycler | Anachem Ltd., Luton, UK |
| Automated DNA extraction | MagNa Pure$^{Tm}$ or MagNa Pure Compact$^{Tm}$ | Roche Diagnostics GmbH, Mannheim Germany |
| Bacterial cell disruption | MagNa- Lyser™ | Roche Diagnostics |
| Centrifuge used for preparing competent cells | Sorvall Evolution RC | Thermo Life Sciences, Basingstoke, UK |
| Nucleic acid preparation | Heating block | Thermo Life Sciences |
| Visualisation and photography of ethidium bromide stained gels | Gel-doc UV transilluminator | Bio-Rad, Hemel Hempstead, UK |
| Homogenisation of food samples | Seward stomacher | Seward Worthing UK |
| Preparing second phase real-time PCR reactions | PCR workstation | Labcaire Systems, Clevedon, UK |
| Gradient PCR | PTC 200 Tetrad thermal cycler | MJ Research Massachusetts, USA |
| Spectrophotometric analysis of purified nucleic acid | Ultrospec | Amersham Pharmcia, Sweden |
| Analysis of sequencing reactions | Beckman Coulter CEQ 8000 Genetic Analysis System. | Beckman Coulter, High Wycombe, UK |
| EMA cross-linking | Osram 500 W Haloline Tungsten Halogen lamp | Halfords, Redditch, UK |
| Incubation of clostridia strains | Anaerobic cabinet | Don Whitley Scentific, Shipley, UK. |
| Incubation of campylobacter strains | Variable atmosphere incubator (VAIN) | Don Whitley Scientific |
| Detection of multiplex generated amplicons | Luminex 100 cytometer | Bio-rad |

## 2.4 Bacterial culture methods

### 2.4.1 General culture conditions

Freeze-dried cultures were reconstituted and cultured according to the NCTC procedures. All strains (NCTC and wild-type), except for campylobacter and clostridia strains, were sub-cultured on Blood Agar, stored at 4 $^{o}$C and used for up to one month unless otherwise stated. All strains, except campylobacter and clostridia, were archived on Nutrient Agar slopes at room temperature (20 $^{o}$C ± 2 $^{o}$C) for the duration of the study.

The campylobacter strains were stored and archived using Microbank beads (Pro-Lab Richmond Hill, Canada) with cryopreservative fluid at -80 $^{o}$C. Clostridia strains were stored and archived using glass beads with 15 % (v:v) glycerol at -20 $^{o}$C. Clostridia strains were cultured on Blood Agar with neomycin (NEOH) and campylobacter cultured on Blood Agar prior to each use.

*Listeria* spp. were incubated for 24 h at 30 $^{o}$C. *Clostridium* spp. were incubated for 24 h in an anaerobic cabinet with an anaerobic atmosphere (80 % $N_2$, 10 % $H_2$, and 10 % $CO_2$). *Campylobacter* spp. were incubated in a variable atmosphere incubator for 48 h in a microaerophillic atmosphere (5 % $H_2$, 3 % $CO_2$, 5 % $O_2$, and 87 % $N_2$). All other bacteria were incubated aerobically at 37 $^{o}$C for 24 h.

### 2.4.2 Aerobic colony count

Bacteria were cultured overnight in Nutrient Broth at 37 $^{o}$C unless stated otherwise. Overnight both cultures were decimally diluted in maximum recovery diluent and cultured on Blood Agar overnight at 37 $^{o}$C following general culture methods applicable to the specific bacterial species used (section 2.4.1). Three agar plates were inoculated per dilution using the spread plate method described by Prescott *et al.* (1999). After incubation, the number

of colonies was counted on each agar plate and the aerobic colony count (ACC) was calculated in colony forming units (CFU) per ml of culture.

### 2.4.3 Culture conditions for *L. monocytogenes* logarithmic growth phase and non-viable cells

For cell viability work, *L. monocytogenes* in the logarithmic growth phase and non-viable cells (killed) were prepared. *L. monocytogenes* was incubated overnight in Brain Heart Infusion (BHI) broth. The overnight broth culture was diluted (1 in 500) in BHI Broth and incubated at 30 $^{o}$C with shaking until an absorbance of 0.3 at 600nm was obtained. The ACC was determined using Blood Nutrient Layered Agar. To prepare non-viable cells, 5 ml of overnight culture was autoclaved for 15 min at 121 $^{o}$C.

### 2.4.4 Culture methods for the detection of *Salmonella* spp., *Campylobacter* spp., *L. monocytogenes*, *S. aureus*, *C. perfringens* and *E. coli* O157:H7 in food samples.

Food samples were analysed following HPA National Standard Methods (Table 2.10). The full methods are available at: http://www.hpa-standardmethods.org.uk/pdf_sops.asp#Food (HPA, 2008).

**Table 2.10 Culture methods used for detection of bacterial pathogens in food samples.**

| Number | Title | Issue date |
|---|---|---|
| F 2 | Preparation of Samples and Dilutions | May 05 |
| F 12 | Enumeration of *Staphylococcus aureus* | May 05 |
| F 13 | Detection of *Salmonella* species | Jul 08 |
| F 14 | Enumeration of *Clostridium perfringens* | May 05 |
| F 15 | Enumeration of *Bacillus cereus* and other *Bacillus* species | May 05 |
| F 17 | Detection of *Escherichia coli* O157 by Automated Immunomagnetic Bead Separation | May 08 |
| F 19 | Detection and Enumeration of *Listeria monocytogenes* & other *Listeria* species | Nov 08 |
| F 21 | Detection of *Campylobacter* species | Aug 08 |

Adapted from HPA (2008).

### 2.4.5 **Food spiking procedure**

Food samples used for spiking were purchased from local supermarkets and used before the manufacturer's "use by" date. All food samples were stored at 4 $^{o}$C. *L. monocytogenes* NCTC 12427 was inoculated into BHI Broth and cultured overnight at 30 $^{o}$C. The culture was then serially diluted in MRD. A 25 g or ml aliquot of the food samples was diluted ten-fold with MRD in a stomacher bag (Seward, London, UK) and homogenised for 30 s at high speed in a stomacher. The food homogenates were spiked by adding 100 µl of appropriately decimally diluted *L. monocytogenes* overnight broth culture to 900 µl of homogenised sample.

## 2.5 Nucleic acid extraction methods

All the buffers used for DNA extraction methods were kit specific and the formulations were confidential.

### 2.5.1 **Sample preparation for MagNa Pure$^{Tm}$ classic and MagNa Pure Compact $^{Tm}$ extraction of bacterial DNA**

Bacterial isolates (Tables 2.1 and 2.2) were taken from solid media and emulsified directly into 300 µl of lysis buffer (Roche Diagnostics GmbH, Mannheim, Germany). The liquid samples (food enrichment broths, broth cultures, culture dilution, spiked samples, serum or CSF) were centrifuged at 6000 *g* for 10 min and the supernatants discarded to leave approximately 50 µl of cell pellet. The lysis buffer was prepared following the manufacturer's instructions and 300 µl of lysis buffer was added to each cell pellet. The sample was then incubated at 65 $^{o}$C for 10 min followed by 95 $^{o}$C for 10 min. DNA was extracted from lysed samples using either the MagNa Pure$^{Tm}$ or MagNa Pure Compact$^{Tm}$ extraction machine. For *L. monocytogenes* culture broth or spiked food homogenate, a 500 µl volume was processed and for serum or cerebral spinal fluid samples, a 1 ml volume was processed.

### 2.5.2 MagNa Pure$^{Tm}$ Classic extraction of total bacterial DNA

A 250 µl aliquot of lysed bacterial cells (section 2.5.1.) was applied to the MagNa Pure$^{Tm}$ extraction machine and DNA extraction was performed according to the manufacturer's instructions supplied with the machine and DNA III kit (Roche Diagnostics). The nucleic acid was eluted into 100 µl of elution buffer.

### 2.5.3 MagNa Pure Compact $^{Tm}$ extraction of total nucleic acid

A 300 µl aliquot of lysed bacterial cells (section 2.5.1.) was applied to the MagNa Pure Compact $^{Tm}$ extraction machine and nucleic acid extraction was performed according to the manufacturer's instructions supplied with the machine and Total Nucleic acid isolation kit (Roche Diagnostics). The nucleic acid was eluted into 100 µl of elution buffer.

### 2.5.4 Nucleic acid isolation using rapid boiling method

Nucleic acid isolation by rapid boiling was performed according to the method of De Medici *et al.* (2003). A 500 µl aliquot of overnight broth culture of *L. monocytogenes* or 500 µl of spiked food homogenate was centrifuged at 14,000 *g* for 10 min. The supernatant was discarded and the pellet resuspended in 150 µl nuclease-free water. The sample was then recentrifuged at 14,000 *g* for 5 min, the supernatant was discarded and the pellet was resuspended in 100 µl of nuclease-free water. The sample was incubated at 100 $^{o}$C for 15 min in a heat block and chilled immediately on ice. Following centrifugation at 14,000 *g* for 5 min, the supernatant was transferred to a tube and incubated at 100 $^{o}$C for 10 min. The sample was then chilled immediately on ice.

### 2.5.5 DNA extraction by modified Boom method

DNA isolation and purification was performed according to the method of McLauchlin *et al.* (1999) with modifications. Bacterial cell disruption was achieved using a MagNa- Lyser™ with MagNa- Lyser™ tubes (Roche

Diagnostics) containing beads, instead of using zircon beads and a bead beater. To lyse the bacterial cells, 900 µl of lysis buffer L6 (Severn Biotech, Kidderminster, UK) and 20 µl of isoamyl alcohol (Sigma, UK) were added to 500 µl of an overnight broth culture of *L. monocytogenes* or 500 µl of a spiked food homogenate in a MagNa- Lyser™ tube containing beads. The mixture was shaken for 1 min in a MagNa- Lyser™ at maximum speed and then centrifuged at 12,000 *g* for 15 s to pellet larger particulate matter. The supernatant was discarded, 100 µl of resuspended silica particles (Severn Biotech) added and the mixture was gently shaken for 10 min to allow the nucleic acid to bind to the silica.

The silica and nucleic acid complex was washed by adding 200 µl of lysis buffer L2, followed by vortexing for 10 min to resuspend the pellet. Samples were then centrifuged at 12,000 *g* for 15 s. The supernatant was discarded and the complex was washed twice in a similar manner with 200 µl of 80 % (v:v) cold ethanol and finally with 200 µl of cold acetone. The sample was then centrifuged at 12,000 *g* for 15 s, the supernatant discarded and the pellet incubated at 56 $^{o}$C for 15 min to remove any residual acetone. DNA was released from the silica by resuspending the pellet in 100 µl of nuclease-free water by incubating at 56 $^{o}$C for 5 min. The silica was pelleted by centrifugation at 12,000 *g* for 15 s and the supernatant containing purified DNA was transferred to a tube. This method is described as 'Boom' in subsequent sections.

### 2.5.6 **DNA isolation using Bugs' n Beads™ bacterial DNA isolation kit**

A Bugs' n Beads™ kit that contained powerlyse reagent and beads and all buffers was purchased from Genpoint (Oslo, Norway). A mix of 20 µl bacterial binding beads and 600 µl of binding and washing buffer was prepared and added to 500 µl of an overnight broth culture of *L. monocytogenes* or 500 µl of spiked food homogenate. The sample was then mixed by pipetting and incubated at room temperature (20 $^{o}$C ± 2) for 5 min to enable the bacteria to bind to the beads. The bacteria and bead complexes were collected using a

magnet and the supernatant discarded using a Pasteur pipette. The sample tube was then separated from the magnet to release the beads. Powerlyse (50 µl) was added to the tube containing the cell pellet and was mixed by inversion to lyse the bacterial cells. To neutralise the powerlyse, 150 µl of cold 95 % (v:v) ethanol was added to the sample, and the beads were resuspended by vortexing. The sample was incubated at room temperature (20 $^{o}$C ± 2) for 5 min to allow the DNA to bind to the beads. The sample tube and magnet were recombined and inverted gently twice to collect all the beads. The supernatant was discarded and the sample tube separated from the magnet to release the beads. The DNA and bead complex was washed by adding 1 ml 70 % (v:v) ethanol to the sample and the tube was briefly vortexed. The magnet and tube were recombined and after the supernatant cleared, the tube was inverted and the supernatant was removed. This step was repeated and then the bead and DNA complex resuspended in 100 µl nuclease-free water. The sample was incubated at 80 $^{o}$C for 10 min to remove any residual ethanol.

### 2.5.7 **Nucleic acid preparation using InstaGene™ matrix**

InstaGene™ matrix (Bio-Rad Hemel Hempstead, UK) was stirred on a magnetic stirring table for 20 min before use. A 500 µl aliquot of a *L. monocytogenes* broth culture or 500 µl of spiked food homogenate was centrifuged at 14,000 *g* for 3 min. The supernatant was discarded and the cell pellet resuspended in 1 ml of nuclease-free water. The sample was recentrifuged at 14,000 *g* for 3 min, the supernatant discarded and the pellet resuspended in 200 µl of InstaGene™ matrix. The sample was incubated at 56 $^{o}$C for 30 min in a heat block, mixed by vortexing and incubated at 100 $^{o}$C for 8 min. The sample was centrifuged at 14,000 *g* for 3 min and the supernatant containing the nucleic acid was decanted to a tube.

### 2.5.8 **Nucleic acid isolation using *micro*LYSIS® DNA release reagent**

A 500 µl aliquot of an overnight broth culture of *L. monocytogenes* or 500 µl of a spiked food homogenate was centrifuged at 14,000 *g* for 10 min. The

supernatant was removed and 19 µl of *micro*LYSIS® reagent (Microzone Ltd, Haywards Heath, UK) was added and the sample mixed by vortexing. The sample was placed in thermocycler and incubated at 65 °C for 5 min, 96 °C for 2 min, 65 °C for 4 min, 96 °C for 1 min, 65 °C for 1 min, 96 °C for 30 s and then cooled to 20 °C. A 5 µl aliquot was used as template DNA in subsequent PCR assays.

### 2.5.9 **Nucleic acid isolation using the NucleoSpin® Food Kit**

NucleoSpin® Food Kit was purchased from Clontech (Palo Alto, USA) all the buffers and columns described in this section were supplied in the kit. A 500 µl aliquot of an overnight broth culture of *L. monocytogenes* or 500 µl of spiked food homogenate was added to 550 µl of buffer CF. This mixture was incubated at room temperature (20 °C ± 2 °C) for 10 min and then at 65 °C for 30 min to lyse the bacterial cells. The sample was centrifuged at maximum speed for 10 min to pellet the cell debris and 300 µl of the supernatant decanted to a fresh tube. The supernatant was mixed with 300 µl buffer C4 and 200 µl of 95 % (v:v) ethanol then vortexed for 30 s. This mixture was applied to a NucleoSpin® food kit column that was centrifuged at 14,000 *g* for 1 min and the eluent discarded.

DNA bound to the column was washed by adding buffer, centrifugation at 14,000 *g* for 2 min and discarding the eluent. The primary wash was performed with 400 µl of buffer CQW, a second wash with 700 µl of buffer C5 and a final wash with 200 µl of buffer C5. The column was placed in a fresh collection tube and DNA was eluted by adding 100 µl of buffer CE at 70 °C, incubating at room temperature (20 °C ± 2 °C) for 5 min and centrifuging at 14,000 *g* for 1 min. The eluted nucleic acid was decanted to a tube.

### 2.5.10 **Nucleic acid isolation using PrepMan™ Ultra sample preparation reagent**

A 500 µl aliquot of an overnight broth culture of *L. monocytogenes* or 500 µl of spiked food homogenate was centrifuged at 6,000 *g* for 10 min and the supernatant discarded. PrepMan™ Ultra reagent (Applied Biosystems Warrington, England) was mixed by vortexing and, after the dispersion of air bubbles, 200 µl was added to the bacterial pellet. The pellet was resuspended by vortexing and incubated at 100 $^{o}$C for 10 min in a heat block. After cooling to room temperature (20 $^{o}$C ± 2 $^{o}$C), the sample was centrifuged at 16,000 *g* for 3 min, the supernatant decanted to a tube and 5 µl used as template DNA in subsequent PCR assays.

### 2.5.11 **Method for investigating MagNa Pure™ as a generic DNA extraction method for foodborne bacterial pathogens**

NCTC cultures of the bacteria species were grown in pure culture on Blood Agar (section 2.4.1). A 20 µl loop of culture was subjected to MagNA Pure™ extraction technique (section 2.5.1 and 2.5.2). A 5 µl aliquot of the resulting DNA was analysed by agarose gel electrophoresis (section 2.15.12) to give an indication of the quality of the extraction.

### 2.5.12 **Agarose gel electrophoresis for the analysis of DNA**

Extracted DNA was analysed by electrophoresis on 0.8 % (w:v) agarose gel (Invitrogen, Paisley, UK) containing 10 mg ml$^{-1}$ of ethidium bromide (Sigma) using 1 x TAE (Invitrogen) running buffer. A 10 µl sample volume was added to the gel that contained 5 µl DNA, 2 µl loading dye (Promega) and 3 µl nuclease-free water. Samples were subjected to electrophoresis for 1 hour at 70 volts. DNA bands were revealed and photographed using Gel-doc UV transillumination and software. A low DNA mass ladder (Invitrogen, Paisley,

UK) was included in each gel. The concentration of the DNA in the sample was calculated using Bionumerics software. A picture of the gel was uploaded into Bionumerics and the intensity of the stained DNA was compared to the DNA mass ladder (Invitrogen). The concentration of the sample DNA was extrapolated by comparison of the sample to the DNA standards in the ladder.

### 2.5.13 **Cell lysis for *S. aureus* using lysostaphin**

A 20 µl loop of *S. aureus* cells was added to 180 µl lysis buffer containing 0.3 mg $ml^{-1}$ lysoszyme (Sigma), 0.03 mg $ml^{-1}$ lysostaphin (Sigma), 1.2 % (v:v) Triton X-100 (Sigma), 2 x TE buffer (20 mM, Sigma) prepared in distilled water. The lysis buffer and cell mix was incubated at 37 $^{o}$C for 30 min and then 25 µl of proteinase K (20 mg $ml^{-1}$, Roche Diagnostics) was added. The mix was incubated at 70 $^{o}$C for 30 min then at 95 $^{o}$C for 10 min. DNA was then extracted from the lysate following the MagNA Pure™ protocol (section 2.5.2)

### 2.5.14 **MagNA lyser cell lysis**

A 20 µl loop of bacterial cells and 240 µl of lysis buffer was added to a MagNa- Lyser™ with MagNa- Lyser™ tubes containing beads (Roche Diagnostics). The mixture was shaken for 1 min in a MagNa- Lyser™ at maximum speed. DNA was then extracted from the lysate following the MagNA Pure™ protocol (section 2.5.2).

### 2.5.15 **RNaqueous RNA isolation technique**

The RNAqueous total RNA isolation kit, (containing buffers 1, 2, 3; wash buffer and elution buffer) was purchased from Ambion (Huntingdon, UK). Bacterial cells were collected from a 1 ml aliquot of a logarithmic phase broth culture of *L. monocytogenes* (section 2.4.3) by centrifugation at 5000 *g* for 5 min at 4$^{o}$C. The supernatant was discarded and pelleted cells were lysed by resuspending in 100 µl TE buffer (Sigma) containing 1 mg $ml^{-1}$ lysozyme (Sigma), incubation at room temperature (20 $^{o}$C ± 2 $^{o}$C) for 5 min and the

addition of 250 µl of lysis/binding solution and mixed by vortexing. To absorb RNA to the membrane, 250 µl ethanol (64 % [v:v], BDH) was added to the lysate and then applied to the column. The sample and column were centrifuged at 8000 *g* for 1 min and the supernatant discarded. RNA bound to the column was washed by addition of the appropriate buffer and centrifugation of the column at 8000 *g* for 1 min and discarding the flow-though. The primary wash was performed using 700 µl of wash buffer 1, then 500 µl of wash buffer 2 and 3 was used for successive washes. RNA was eluted by subsequent additions of 40 µl, 10 µl and 50 µl of elution buffer and centrifugation at 8000 *g* for 1 min.

### 2.5.16 **RNeasy RNA isolation technique**

An RNeasy RNA extraction kit containing buffer RLT, RPE and RW1 and RNase-free DNase I enzyme set containing buffer RDD were purchased from Qiagen (Crawley, UK). Bacterial cells were collected from a 1 ml aliquot of BHI Broth culture of *L. monocytogenes* by centrifugation at 5000 *g* for 5 min at 4$^{o}$C. The supernatant discarded and pelleted cells were resuspended in 100 µl of 1 x TE buffer (Sigma) containing 3 mg ml$^{-1}$ lysozyme (Sigma) and incubated at room temperature (20 $^{o}$C ± 2 $^{o}$C) for 10 min to lyse the cell walls. The lysate was mixed with 450 µl buffer RLT by vortexing and then centrifuged at maximum speed for 2 min. The eluent containing RNA was collected and 250 µl ethanol was added before applying to the RNeasy column. Supernatant was removed by centrifugation for 8000 *g* for 15 s. The bound RNA was washed by adding 350 µl of buffer RW1, centrifugation at 9000 *g* for 15 s and discarding the eluent.

DNase I enzyme was prepared by adding 10 µl of the enzyme to 70 µl of buffer RDD and mixed by inversion. DNA in the sample was digested by applying 80 µl DNase I enzyme mix to the column and incubating at room temperature (20 $^{o}$C ± 2 $^{o}$C) for 15 min. The bound RNA was washed by adding 350 µl of appropriate buffer, centrifugation at 8000 *g* for 15 s and discarding the eluent. The primary wash was performed with buffer RW1, and

a second wash with buffer RPE. To eliminate buffer RPE carry over, the column was centrifuged at 9000 *g* for 15 s, then for 1 min and eluent discarded. The RNA was eluted by applying 50 µl RNase-free water to the column and then centrifuged at 9000 *g* for 1 min.

### 2.6 Construction of *E. coli* GFP

The strains used and constructed, their source and culture conditions are detailed in Table 2.11.

**Table 2.11 Bacterial strains and plasmids for construction of *E. coli* GFP.**

| Strain | Designation | Source | Selective marker |
|---|---|---|---|
| *E. coli* CC118 λ pir pAG408 (ATCC 87653) | Source of plasmid pAG408 | Laboratory of the Government Chemist, (Teddington, UK) | Ampicillin (50 µgml$^{-1}$, Sigma) and kanamycin (20 µgml$^{-1}$, Sigma) |
| *E. coli* S17- λ pir | | Biomedal, (Seville, Spain) | Streptomycin (30 µgml$^{-1}$, Sigma) |
| *E. coli* S17- λ pir pAG 408 | Donor | This study | Kanamycin (20 µgml$^{-1}$, Sigma) and streptomycin (30 µgml$^{-1}$, Sigma) |
| *E. coli* BLR | Recipient | Merck (KGaA, Darmstadt, Germany) | Tetracycline (12.5 µgml$^{-1}$, Sigma) |
| *E. coli* GFP | GMO | This study | Kanamycin (20 µgml$^{-1}$, Sigma) and tetracycline (12.5 µgml$^{-1}$, Sigma) |

All experiments were conducted in LB Broth or Agar as required.

#### 2.6.1 Isolation of plasmid pAG408 from *E. coli* CC118 λ pir by plasmid mini kit

The *E. coli* strain CC118 λ pir ATCC 87653 contained the plasmid pAG408. The plasmid was isolated with a kit that used the modified alkaline lysis procedure. To extract the plasmid, an overnight culture of LB Broth containing 50 µgml$^{-1}$ ampicillin (Sigma) (marker on strain) and 20 µgml$^{-1}$ kanamycin (Sigma) (marker on plasmid) were set up from a single colony of *E. coli* strain

CC118 λ pir ATCC 87653 selected from a freshly streaked LB Agar plate. The culture was incubated for 8 h at 37 °C with vigorous shaking (300 rpm). This culture was then diluted to $10^{-3}$ in 3 ml of selective LB broth and incubated for 16 h at 37 °C with vigorous shaking (300 rpm). The bacterial cells were collected by centrifugation at 6,000 *g* for 15 min at 4 °C.

A plasmid mini kit containing all the required buffers (designated P1, P2, P3, QBT, QC and QF) and Qiagen-tip 20 were purchased from Qiagen. The supernatant from the overnight culture of *E. coli* strain CC118 λ pir ATCC 87653 collected by centrifugation was decanted and the bacterial pellet was resuspended in 300 µl of buffer P1. The bacteria were lysed by adding 300 µl of buffer P2 mixing by inversion and incubating at room temperature (20 °C ± 2) for 5 min. The genomic DNA, protein and cell debris were precipitated by adding 300 µl of chilled buffer P3, mixed by inversion and incubated on ice for 5 min.

The suspension was centrifuged at 14,000 *g* for 10 min and the supernatant removed to a fresh tube. A Qiagen-tip 20 was equilibrated by applying 1 ml of buffer QBT to the column; once the buffer had passed through the column the supernatant containing the plasmid DNA was added. Bound plasmid DNA was washed twice with 2 ml of buffer QC and eluted with 800 µl of buffer QF. The plasmid DNA was precipitated by adding 560 µl of isopropanol (20 ± 2 °C), mixed by vortexing and then centrifuged at 14,000 *g* for 30 min. The supernatant was decanted and the plasmid pellet washed with 1 ml 70 % ethanol (v:v). The pellet was air-dried for 10 min and the plasmid DNA resuspended in 50 µl of 1 x TE buffer (Sigma). The plasmid DNA concentration was determined by UV spectrophotometry at 260 nm as described by Sambrook *et al.* (1989). The plasmid DNA was stored at 4 °C until used.

### 2.6.2 **Preparation of competent *E. coli* S17- λ pir cells**

Competent cells were prepared using the calcium chloride protocol described by Sambrook *et al.* (1989). A single colony of *E. coli* S17-1 λ pir (Biomedial, Seville, Spain) was transferred to 100ml of LB Broth containing streptomycin (30 $\mu g ml^{-1}$) and was incubated for 3 h at 37 $^{o}$C with vigorous shaking (300 rpm). The culture was transferred to sterile ice-cold tube and incubated on ice for 10 min. The cells were collected by centrifugation at 2500 *g* for 10 min at 4 $^{o}$C. The supernatant was removed and the pellet resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ (Sigma). The cells were collected by centrifugation as above and the pellet resuspended in 2 ml ice-cold 0.1 M $CaCl_2$ (Sigma). Competent cells were stored at 4 $^{o}$C for 24 h prior to transformation.

### 2.6.3 **Transformation of competent *E. coli* S17- λ pir cells with pAG408**

Competent *E. coli* S17- λ pir cells were transformed with pAG408 plasmid DNA using the protocol described by Sambrook *et al.* (1989). Plasmid DNA (45 ng) was added to a 200 µl of competent *E. coli* S17- λ pir cells. The tubes were mixed by swirling gently and incubated on ice for 30 min. The tubes were transferred to a circulating water bath at 42 $^{o}$C and incubated for 90 s. The cells were cold shocked in ice for 2 min and then incubated at 37 $^{o}$C for 45 min with 800 µl of Super optimal catabolite repression (SOC) medium (Sigma). Transformants were selected on LB Agar containing kanamycin (20 $\mu g ml^{-1}$) and streptomycin (30 $\mu g ml^{-1}$) following incubation overnight at 37 $^{o}$C.

### 2.6.4 **Restriction endonuclease digest and gel electrophoresis of plasmid DNA**

Restriction endonuclease digestion was performed using 0.2 µg plasmid DNA, 1 x buffer (Invitrogen), 2.5U *Kpn*I (Invitrogen) and nuclease-free water in a 10 µl volume. The digest mixture was incubated at 37 $^{o}$C for 90 min and then the enzyme inactivated by incubating at 65 $^{o}$C for 10 min. A 5 µl aliquot of digested plasmid fragments was separated by electrophoresis for 1 h at 100V on a 0.8 % (w:v) agarose (Invitrogen) gel containing 10 mg $ml^{-1}$ ethidium bromide (Sigma) using 1 x TAE running buffer (Invitrogen). The plasmid

fragments were visualised and photographed using Gel-doc UV transillumination and software. Fragment size was estimated by comparison to λDNA/*Hin*dIII fragments (Invitrogen).

### 2.6.5 **Filter mating of *E. coli* S17-1 λ pir containing pAG408 and *E. coli* BLR**

Filter mating was performed as described by Sasaki *et al.* (1988). Briefly, donor cells (*E. coli* S17-1 λ pir containing pAG408) were cultured in LB Broth with kanamycin (20 μg $ml^{-1}$, Sigma) and streptomycin (30 μg $ml^{-1}$, Sigma) overnight at 37 $^{o}$C with shaking. Recipient cells (*E. coli* BLR Merck KGaA, Darmstadt, Germany) were cultured in LB Broth with tetracycline (12.5 μg $ml^{-1}$) overnight at 37 $^{o}$C with shaking. Equal volumes of overnight broth cultures (2.5 ml) of donor and recipient cells were added to a 5 ml syringe and mixed by inversion. The mixed culture was then applied by syringe to sterile HA membrane filters (0.45 μM pore size, 25mm diameter Millipore, Watford, UK) contained in swinnex filter holders (Millipore). Three filters were prepared and each was placed on LB Agar plate and incubated at 37 $^{o}$C overnight. The growth on the filters was washed off with 1.5 ml Buffered Peptone Water and the cells collected by centrifugation for 2 min at 8,000 *g*. The supernatant was removed and the resulting cells cultured on LB Agar with kanamycin (20 μg $ml^{-1}$) and tetracycline (12.5 μg $ml^{-1}$) at 37 $^{o}$C for 48 h. Colonies of kanamycin-resistant transconjugants were then examined for fluorescence following excitation with a 366-nm UV lamp and photographed using a Cannon PowerShot A50 camera.

### 2.6.6 **Lenticule disc preparation**

Primary Lenticule discs encapsulating the *E. coli* GFP strain were prepared in collaboration with the Food and Environmental Proficiency Testing Unit (FEPTU, HPA CFI, London, UK) as described by Codd *et al.* (1998). *E. coli* GFP was cultured on MacConkey Agar at 37 $^{o}$C for 24 h. One colony was emulsified in 500 μl of Nutrient Broth and used to inoculate a 30 ml nutrient

Agar slope, which was incubated at 37 $^{o}$C for 24 h. Growth from the slope was collected and emulsified in 500 µl of Nutrient Broth, then added to 2.5 ml of lenticulation fluid (FEPTU) containing Ponceau S dye (33.2 µg ml$^{-1}$, Merck, Hoddesdon, UK). The inoculated fluid was dispensed in 25 µl volumes onto Parafilm (VWR International Lutterworth, UK). The fluid aliquots were stored for nine days at 4 $^{o}$C and two days at -25 $^{o}$C until discs had formed. The Lenticule discs were then transferred to a screw capped vial containing a silica desiccant and stored at 4 $^{o}$C for up to four months.

Secondary Lenticule discs were prepared by resuspending 1 primary disc in 1 ml of Pages saline and then 378 µl of this was transferred to 25 ml of lenticulation fluid and the sample mixed by inversion. The discs were aliquoted, dried and organisms per disc enumerated as described previously for the primary Lenticules.

### 2.6.7 **Milk spiking with *E. coli* GFP**

*E. coli*-BLR GFP was cultured overnight in Nutrient Broth at 37 $^{o}$C. A ten-fold serial dilution was prepared in MRD. Six 9 ml aliquots of semi-skimmed milk were inoculated with (1 ml) one of each *E. coli*-BLR GFP serial dilution. The ACC was determined using LB Agar and prepared in triplicate (section 2.4.2).

## 2.7 RNA detection methods

### 2.7.1 Reverse transcritption real-time PCR

For comparison of the one step and two step RT-PCR assays 5 µl nucleic acid was tested in duplicate the one-step RT-PCR (section 2.7.2) or two-step RT-PCR (section 2.7.3) following the manufacturer's instructions. The GADPH target was supplied in the kit and diluted in 1 x TE buffer for comparisons of the one-step and two-step RT-PCR. For detection of *L. monocytogenes* mRNA 5 µl nucleic acid was tested in duplicate the one-step RT-PCR using the *L. monocytogenes* hlyAF and hlyAR oligonucleotides (Table 2.6) and hlyA probe (Table 2.7). The altered oligonucleotide RT-PCR was performed using hlyAF and RTHlyA oligonucleotides and the one-step RT-PCR.

### 2.7.2 One step real-time RT-PCR reaction

The one step real-time PCR was performed using TaqMan Gold RT-PCR kit (Applied Biosystems), which contained all reagents except *L. monocytogenes* specific oligonucleotides and probe. The one step RT-PCR contained: 1 µl nucleic acid, 5 µl 10x TaqMan buffer A 5.5 mM $MgCl_2$ , 300 µM dATP, dCTP and dGTP; 600 µM dUTP, 100 nM FAM labelled hlyA probe, 200 nM oligonucleotide hlyAF and hlyAR 0.025 U AmpliTaq Gold DNA polymerase, 0.025 U MultiScribe Reverse transcritpase and 0.08 U RNase inhibitor in a 50 µl reaction volume. The reaction conditions consisted of a 48 $^{o}$C hold for 30 min and 95 $^{o}$C for 10 min and then 40 cycles of 95 $^{o}$C for 20 s and 60 $^{o}$C for 1 min. The assay was performed in an ABI Prism 7000 or 7700 sequence detection system.

### 2.7.3 Two step real-time RT-PCR reaction

The two-step real-time PCR was performed using TaqMan Gold RT-PCR kit (Applied Biosystems), which contained all reagents and except *L. monocytogenes* specific oligonucleotides and probe. The two-step RT-PCR consisted of a reverse transcription phase, followed by a PCR amplification

phase. The reverse transcription reaction mix consisted of: 1 µl nucleic acid, 5 µl 10x TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 200 nM hlyAR oligonucleotide, 0.08 U RNase inhibitor, 1.25 U Mulitscribe reverse transcriptase in a 10 µl reaction volume. The reverse transcription cycling conditions consisted of a 25 $^oC$ hold for 10 min, then a 48 $^oC$ hold for 30 min and finally 95 $^oC$ for 15 s. The reaction mix (10 µl) from the reverse transcription was added to the PCR reaction mix. The PCR reaction mix contained: 10 µl mix from reverse transcription phase, 5 µl 10x TaqMan buffer A, 11.0 mM $MgCl_2$, 100 µM dATP, dCTP and dGTP; 600 µM dUTP, 100 nM FAM labelled hlyA probe, 100 nM oligonucleotide hlyAF and hlyAR 0.25 U AmpliTaq Gold DNA polymerase, 0.5 U Amperase in a 50µl reaction volume. The reaction conditions consisted of a 50 $^oC$ hold for 2 min and 95 $^oC$ for 10 min and then 40 cycles of 95 $^oC$ for 15 s and 60 $^oC$ for 1 min. The assay was performed in an ABI Prism 7000 or 7700 sequence detection system.

### 2.7.4 **Nucleic acid sequence based amplification (NASBA)**

Nucleic acid (5 µl) was extracted using MagNa Pure Compact and Total nucleic kit and then tested in duplicate in all NASBA reactions. The following NASBA reactions were used: Standard NASBA (section 2.7.5), Real-time NASBA (section 2.7.6) and the kit-based Nucliesens NASBA (section 2.7.7). The modification for amplifying DNA targets in the NASBA reaction is described in section 2.7.8. Oligonucleotides P1, (or P1T7), P2 the molecular beacon used for the real-time NASBA were designed by www.premierbiosoft.com using Beacon designer™ software. Oligonucleotides P1, (or P1T7) in combination with P2 amplified a 222 bp fragment of the distal section of the *L. monocytogenes hlyA* gene. Oligonucleotides BTP1 and BTP2 were selected to amplify a 134 bp fragment of the *L. monocytogenes hlyA* gene.

### 2.7.5 **Standard NASBA for the detection of *L. monocytogenes hlyA* gene mRNA target**

The NASBA buffer was prepared and contained 100 mM Tris-HCl pH 8.5 (Ambion), 30 mM $MgCl_2$ (Invitrogen), 125 mM KCl (Ambion), 2.5 mM of each dNTP (Invitrogen), 5 mM each of ATP (Ambion), UTP (Ambion), CTP (Ambion), and GTP (Ambion). The oligonucleotide mix consisted of 15 % (v:v) DMSO (Sigma) and 5 pmol of each of the BTP1 and BTP2 oligonucleotides. A reaction mix was prepared and contained 10 µl of NASBA buffer, 6.25 µl of oligonucleotide mix, 10 mM DTT (Sigma) and 5 µl of template was added. The reaction mixture was incubated at 65°C for 5 min and cooled to 41°C for 5 min. Once the mixture had equilibrated to 41°C, 2 µl of enzyme mix was added. The enzyme mix consisted of 375 mM sorbitol (Sigma), 1.3 µg BSA (Sigma), 0.2 units *E. coli* RNase H (Invitrogen), 40 units T7 RNA polymerase (Amersham) and 8.0 U AMV-reverse transcriptase Seigakaku (Japan). The total reaction volume was 25µl. Reactions were then incubated at 41°C for 90 min. The assay was performed in a T3 Biometra Thermocycler.

### 2.7.6 **Real-time NASBA for the detection of *L. monocytogenes hlyA* mRNA target**

The real-time NASBA reaction contained premix, buffer and oligonucleotide mixes. The premix consisted of 4 µl water, 6 ng NASBA beacon and 20 pmol ROX (Insight Biotechnology, Wembley, UK). The NASBA buffer consisted of 200 mM Tris-HCl pH 8.5 (Ambion), 60 mM $MgCl_2$ (Invitrogen), 350 mM KCl (Ambion), 2.5 mM DTT (Sigma), 5 mM each dNTP (Invitrogen), 10 mM ATP (Ambion), UTP (Ambion) and CTP (Ambion), 7.5 mM GTP (Ambion) and 2.5 mM ITP(Ambion). The oligonucleotide mix consisted of 7.5 mM ATP, UTP and CTP, 7.5 mM GTP and 1 µM each of P1T7 and P2. A reaction mix was prepared that contained 6 µl of premix, 4 µl of NASBA buffer and 4 µl of oligonucleotide mix and 1 µl of template was added. The reaction mixture was incubated at 65°C for 5 min and cooled to 41°C for 5 min in a T3 Biometra thermocycler. Once the mixture had equilibrated to 41°C, 5 µl of enzyme mix

was added, which contained 375 mM sorbitol (Sigma), 2.1 µg BSA (Sigma), 0.08 U RNase H (Invitrogen) , 32 U T7 RNA polymerase (Amersham) and 6.4 U AMV-reverse transcriptase Seigakaku (Japan). The total reaction volume was 25µl. Reactions were then incubated at 41°C for 90 min. The assay was performed in an ABI Prism 7000 or 7700 sequence detection system.

### 2.7.7 **NuclieSENS NSABA reaction**

A NucliSENS NASBA reaction was purchased from Organon Teknika (Boxtel, The Netherlands) and contained all the regents except: ROX, oligonucleotides and probes. The NucliSENS NASBA reactions were performed in a total volume of 20 µl containing 5 µl of nucleic acid, 5 µl of enzyme mix, and 10 µl of amplification mix, prepared as described in the NucliSENS Basic Kit manual. 6ng of NASBA beacon and 20 pmol of ROX (Insight Biotechnology) were added to the amplification mix to allow real-time detection. The enzyme mixture contained T7 RNA polymerase, avian myeloblastosis virus reverse transcriptase, RNase H, and bovine serum albumin and was added to the reaction after heat denaturation of the target RNA (5 min at 65 °C). The reaction mixtures were then incubated for 90 min at 41 °C in a T3 Biometra thermocycler. Amplicons were analysed using gel electrophoresis (section 2.9.4).

### 2.7.8 **DNA NASBA modification**

The NASBA reaction described in section 2.9.4 was modified from amplifying an RNA target to a DNA target. A new oligonucleotide P2T7 was designed for the DNA target and a heating step to melt the DNA and allow the oligonucleotide to bind was added. The DNA was heated to 95 $^{o}$C for 5 min with the P2T7 (Table 2.6). The primed DNA was added to the real-time NASBA reaction.

## 2.8 Ethidium monoazide methods

Ethidium monoazide (Invitrogen) 8-azido-3-amino-5-ethyl-6-phenylphenan thridinium chloride (EMA) is a light sensitive chemical and so was stored in the dark until used.

### 2.8.1 Bacterial culture EMA DNA cross linking method

EMA cross-linking of DNA was performed as described by Nogva *et al.* (2003). A 1 ml aliquot of *L. monocytogenes* NCTC 12427 overnight BHI broth culture was centrifuged at 10000 *g* for 7 min and the supernatant discarded. Pelleted cells were resuspended in 1 ml EMA (100 $\mu l ml^{-1}$). The resuspended cells were placed on ice and incubated for 5 min in black microfuge tubes (Anachem, Luton, UK). The sample was transferred to a clear tube, replaced on ice, 20 cm from the light source and incubated for 2 min. The cells were pelleted by centrifugation at 10,000 *g* for 30 s. The pellet was washed twice by adding 500 µl nuclease free water, resuspending the cells by briefly vortexing and then centrifuged at 10,000 *g* for 30 s and finally the supernatant was discarded.

### 2.8.2 EMA DNA cross linking method for optimisation experiments

A 10 µl aliquot of *L. monocytogenes* NCTC 12427 DNA and 1 ml EMA (100 $\mu l\ ml^{-1}$) were placed in a black microfuge tube (Anachem). The sample was placed on ice and incubated for 5 min. This was then transferred to a clear tube, replaced on ice 20 cm from the light source and incubated for 2 min. DNA was precipitated from an EMA-treated sample by the addition of 2 ml of ice-cold ethanol (95 % [v:v]) and centrifuged at 4 $^{o}C$ for 30 min at full speed. The supernatant was removed, 500 µl of ice-cold ethanol (70 % [v:v]) was added and the sample was re-centrifuged for 5 min at maximum speed. This step was repeated with a 3 min centrifugation. The supernatant was removed and the pelleted DNA air-dried. The DNA was then re-suspended in 100 µl of nuclease-free water.

### 2.8.3 **Application of EMA DNA cross-linking to *Taq* Polymerase**

All plastic consumables, pipettes, $MgCl_2$ (Invitrogen), PCR buffer A (Applied Biosystems) and nuclease-free water were exposed to UV light for 10 min in a PCR workstation following manufacturer's instructions for decontamination. Working solutions of dNTPs and EMA were prepared with UV-treated nuclease-free water before each experiment. An aliquot of EMA at a concentration of 200 µl $ml^{-1}$ and 0.25 µl Taq DNA polymerase (Invitrogen) were added to a black microfuge tubes. The sample was placed on ice, incubated for 10 min, and then transferred to a clear tube. The sample was then replaced on ice 20 cm from the 100W fluorescent lamp and incubated for 60 s. The Taq DNA polymerase was then used in a 16S rDNA PCR (section 2.9.6).

## 2.9 PCR amplification methods

### 2.9.1 Design of PCR assays

GenBank sequences were collected for the target gene and aligned using Megaline software version 5.01 (DNAStar, Madison WI, USA). A consensus sequence was produced by MegaLine that was 100% homologous to all the sequences was selected. The consensus sequence was applied to Primer Express version 2.1 software (Applied Biosystems), which designed a real-time PCR using TaqMan chemistry (i.e. using a hydrolysis probe).

The oligonucleotide and probe design by the software to comply with the following recommendations: a melting temperature ($T_m$) of 58-60 $^oC$, guanine (G) to cytosine (C) 20-80 %, 9-40 nucleotides in length, < 2 $^oC$ difference in $T_m$ between the two oligonucleotides and maximum of 2/5 G or C at 3' end. The probe design complied with the following recommendations: $T_m$ 10 $^oC$ above oligonucleotides, G to C ratio of 20-80 %, up to 40 nucleotides in length, and less than 4 continuous Gs. The amplicon was 50-150 base pairs in length and reverse oligonucleotide was as close to the probe as possible without over lapping.

Primer Express designed up to 200 oligonucleotide and probe combinations. A probe was selected that contained more guanine than cytosine bases as recommended by the manufacturer. A probe and oligonucleotide combination was selected which did not share 100% sequence homology with sequences other than that of the target gene, which was assessed using BLASTN software. The oligonucleotide $T_m$ was calculated using Primer Express with salt concentration of 0.55 mM salt and 0.3 µl oligonucleotides.

### 2.9.2 Bacterial culture and DNA extraction for validation of PCR assays

For PCR specificity pure bacterial cultures were grown on Blood Agar (section 2.4.1) then a 20 µl loop of culture was used for DNA extraction (section 2.5.1 - 2.5.2). For PCR sensitivity measurements pure cultures of NCTC bacterial

strains were used as described in section 2.1.1. The strains were cultured on Blood Agar (section 2.4.1) then an isolate used to inoculate a broth that was incubated overnight. For PCR sensitivity measured in CFU the overnight broth culture was ten-fold serially diluted in MRD and aliquots of the dilutions for ACC calculations (section 2.4.2) and for DNA extraction (section 2.5.1 -2.5.2). The extracted DNA was then applied to the PCR (section 2.9.7 or 2.9.8). For PCR specificity measured in genome copies the overnight culture was subjected to DNA extraction (section 2.5.1 -2.5.2) then the DNA was quantified by spectrophotometry (section 2.9.14). The quantified DNA was then ten-fold serially diluted and applied to the PCR (section 2.9.7 or 2.9.8).

### 2.9.3 **Conventional PCR**

PCR amplification for the detection of target pathogens was performed using puRETaq Ready-To-Go$^{TM}$ PCR Beads (Amersham), 5 µl DNA, and 2.5nM of each forward and reverse or reverse external oligonucleotide (Table 2.6) in a 25 µl reaction volume. Oligonucleotide choice was dependent on which assay was run and is described in the results section. The cycling conditions were 94 $^{o}$C for 2 min, followed by 25 cycles: 94 $^{o}$C for 20 s, 55 $^{o}$C for 20 s followed by 72 $^{o}$C for 30 s. The assay was performed in a T3 Biometra thermocycler.

### 2.9.4 **Gel electrophoresis of amplicons**

Amplicons were analysed by electrophoresis on either 2 % (w:v) (amplicon size ≥ 200 bp) or 4 % (w:v) (amplicon size ≤ 200 bp) E-gels (Invitrogen). A 5 µl aliquot of amplified product was mixed with 15 µl of nuclease-free water and 2 µl of 6x blue/orange loading dye (Promega) and 20 µl were loaded onto the gel. The amplicons were subjected to electrophoresis at 70 V for 20-30 min. Amplicons were revealed and photographed using Gel-doc UV transillumination and software. A 100 bp DNA ladder was included in each gel to estimate the amplicon size.

### 2.9.5 **Real-time PCR**

All real-time PCRs were performed in ABI Prism 7000 or 7700 sequence detection system (SDS). All the reactions were performed in duplicate for each sample. In each PCR run a positive control consisting of 5 µl of target template and negative control consisting of 5 µl nuclease-free water were also tested.

The fluorescent signal was measured by the instrument during the exponential phase of the PCR. $\Delta R_n$ is a measure of the magnitude of the fluorescent signal given by a set of PCR conditions, which is calculated as the $R_n$ of the template and all components of the reaction minus the $R_n$ of the sample in the early cycles (baseline) prior to a detectable increase in fluorescence. A threshold that intersects the amplification plot in the linear region of the plot was then applied manually that gave the threshold cycle ($C_T$). The $C_T$ value was defined as the PCR cycle number at which an increase in fluorescence is first detected for each amplification plot. The Applied Biosystem SDS instrument SDS v1.2 software automatically calculated the CT and $\Delta R_n$ values.

### 2.9.6 **16S rDNA amplification.**

The real-time PCR for the amplification of 16S rDNA contained 5 µl of DNA, 5 mM $MgCl_2$ (Applied Biosystems), 1 X buffer (Invitrogen), 0.125 U *Taq* DNA polymerase (Invitrogen), 200nM of each dATP (Applied Biosystems), dCTP (Applied Biosystems), dGTP (Applied Biosystems) and dTTP (Applied Biosystems); 200 nM of each uniF and uniR oligonucleotides; 100 nM FAM labelled uni probe, 8 nM ROX (Insight Biotechnology) in a 25 µl reaction. The cycling conditions consisted of a 50 $^oC$ hold for 2 min, 95 $^oC$ for 10 min and then 45 cycles of 95 $^oC$ for 20 s followed by 60 $^oC$ for 1 min.

### 2.9.7 **Conditions for monoplex real-time 5' nuclease PCR assays**

The oligonucleotide and probe sequences are provided in Table 2.6 and Table 2.7 for all the real-time PCR assays described in this study. The monoplex

PCR assays contained 12.5 µl qPCR mastermix, 5 µl of template DNA, 300 nM of each forward and reverse oligonucleotide and 100 nM probe in a 25 µl reaction. The qPCR mastermix (Eurogentec, Seraing Belgium) contained dNTPs (including dUTP), hot Goldstar DNA polymerase, $MgCl_2$ (5 mM final concentration), uracil-N-glycosylase (UNG), stabilisers and ROX passive reference dye. The cycling conditions consisted of a 50 $^{o}$C hold for 2 min, 95 $^{o}$C for 10 min and then 40 cycles of 95$^{o}$C for 20 s followed by 60 $^{o}$C for 1 min. Oligonucleotides designated gene name F and R in combination with a TaqMan probe for the target gene appropriate to the microorganism being assayed. For example, to detect *S. enterica iroB* gene by real-time PCR the oligonucleotides iroBF and iroBR were used in combination with probe iroB. Oligonucleotides and their target gene and organism are given in Table 2.6. Probes and their target gene and organism are given in Table 2.7. All reactions used probes concentrations described above except iroB probe concentration that was 30 nM per reaction.

### 2.9.8 **Internal positive control 5'nuclease real-time duplex PCR assay**

The internal positive control (IPC) (Applied Biosystems) kit contained IPC oligonucleotides and a VIC labelled probe mix, IPC DNA and blocker. The PCR was performed as described by the manufacturer's instructions. Each duplex PCR contained 12.5 µl qPCR mastermix (Eurogentec), 5 µl template DNA, and 0.75 nM forward and reverse oligonucleotide and 2.5 mM FAM labelled probe, 2.5 µl IPC mix and 0.5 µl IPC DNA in a 25 µl reaction. A non-amplification control (NAC) that contained of 5 µl IPC blocker in place of template DNA and included in each experiment. The amplification conditions consisted of a 50 $^{o}$C hold for 2 min, 95 $^{o}$C for 10 min followed by 40- 45 cycles of 95 $^{o}$C for 20 s and 60 $^{o}$C for 1 min. The IPC was run as a duplex with all of the 5'nuclease real-time PCR assays except the 16S rDNA. Oligonucleotides and probe choice for target pathogens was as described in section 2.9.7.

### 2.9.9 **Process control 5'nuclease real-time duplex PCR assay**

The duplex process control PCR assays contained 12.5 µl qPCR mastermix, 5 µl of template DNA, 300 nM of each forward and reverse oligonucleotide for the target pathogen and 100 nM FAM labelled probe for the target. This was combined with the gfp reaction that contained 100nM of VIC labelled probe specific for the gfp target and 50nM and 200 nM concentrations of forward and reverse gfp oligonucleotides per reaction respectively. The total reaction volume was 25 µl. The qPCR mastermix (Eurogentec, Seraing Belgium) contained dNTPs (including dUTP), hot Goldstar DNA polymerase, $MgCl_2$ (5 mM final concentration), uracil-N-glycosylase, stabilisers and ROX passive reference dye. The cycling conditions consisted of a 50 $^{o}$C hold for 2 min, 95 $^{o}$C for 10 min and then 40 cycles of 95$^{o}$C for 20 s followed by 60 $^{o}$C for 1 min. Oligonucleotides and probe choice for target pathogens was as described in section 2.9.7.

All assays used the standard conditions described above except: the *C. coli* PCR used a gfp probe concentration of 150 nM and the ceuE probe concentration of 50 nM per reaction. The *L. monocytogenes* PCR *hlyA* probe concentration was 200 nM per reaction. The *C. jejuni* PCR gfp probe concentration was 125 nM per reaction. The *S. enterica* PCR used a iroB probe concentration of 35 nM per reaction.

### 2.9.10 **Nested and hemi-nested PCR assay design**

The nested PCR assay was designed using Primer Express software 2.1 using default conditions (section 2.9.1). The hemi-nested PCR oligonucleotides were designed manually, following the recommendations: 19-21 bases in length, 50 % G:C ratio, with the two 3' bases and the 5' base being G's or C's, no imbalance of GC content along the oligonucleotide or long runs (i.e. greater than four) of a single base and a melting temperature of approximately 60°C (calculated as 2x adenine (A) plus thiamine (T) + 4xG+C).

### 2.9.11 **Hemi-nested PCR**

Hemi-nested PCR assays used conventional PCR conditions (section 2.9.3). The oligonucleotides used for first phase PCR were suffixed with F and RX. For example, to detect *S. enterica* iroB gene the oligonucleotides iroBF and iroBRX were used. Oligonucleotides with their target gene and organism are given in Table 2.6. PCR amplicons from the first phase of the nested PCR were diluted ten-fold in nuclease-free water and 5 µl used as template in the real-time PCR using (section 2.9.7).

### 2.9.12 **Nested PCR assay for the amplification of *L. monocytogenes hlyA* gene**

The first phase PCR amplification was performed using puRETaq Ready-To-Go™ PCR Beads (Amersham Bioscience), 5 µl target DNA, and 2.5 nM of each oligonucleotide hlyOF and hlyOR in a 25 µl reaction volume. PCR amplifications were performed in a T3 Biometra thermocycler and the cycling conditions were 94 $^{o}$C for 2 min, followed by 25 cycles: 94 $^{o}$C for 20 s, 55 $^{o}$C for 20 s and 72 $^{o}$C for 30 s. PCR amplicons from the first phase of the nested PCR were diluted ten-fold in nuclease-free water and 5 µl used as template in the real-time PCR using hlyAF and hlyAR oligonucleotides (section 2.9.7).

### 2.9.13 **RNase digestion and Qiaex DNA purification**

DNA purification was performed with Qiaex kit (Qiagen) that contained the Qiaex reagent, buffers QX1 and PE. DNA was incubated with 10 µg ml$^{-1}$ RNase (Sigma) at 37 $^{o}$C for 30 min using a heating block. Three volumes of buffer QX1 were added to one volume of RNase treated DNA. Qiaex II reagent was resuspended by vortexing for 30 s and 10 µl added to the DNA sample. This mixture was then incubated at room temperature (20 $^{o}$C ± 2) for 10 min and mixed every 2 min by inversion. The sample was centrifuged at 14,000 *g* for 30 s to pellet the DNA. The supernatant was removed and the pellet washed twice with 500 µl of buffer PE (Qiagen). The pellet was air-dried by incubating at room temperature (20 $^{o}$C ± 2) for 15 min or until the

pellet became white. The pellet was resuspended in 50 µl nuclease-free water and centrifuged at full-speed for 30 s. The supernatant containing DNA was decanted to a fresh tube.

### 2.9.14 **Quantification of nucleic acid by spectrophometry**

Nucleic extracts were diluted 100-fold in 1 x TE buffer (Sigma) and absorbance was measured at 230, 260, 280 and 320 nm in Uvettes (UV cuvettes, Helens BioScience, Sunderland UK) with a 10 mm cell path. The Ultrospec automatically calculated nucleic acid concentrations.

## 2.10 Statistical and mathematical calculations

### 2.10.1 Calculation of the molecular weight of a bacterial genome

The mass of one genome (femtograms) was calculated using the following:

The number of nucleotides in one genome x the mean molecular weight of a DNA base pair divided by Avogadro's constant ($6.022 \times 10^{23}$). The weight of one genome for the target organism used in this study is given in Table 2.12.

**Table 2.12 Molecular weight of one genome for target organisms used in this study**.

| Bacterial species | NCTC number | Weight of one genome (fg) | Approximate genome size in base pairs (Reference) |
|---|---|---|---|
| *C. coli* | n/a | 1.82 | 1,680,000 (Fouts *et al.,* 2005) |
| *C. jejuni* | NCTC 11168 | 1.80 | 1,641,481 (Parkhill *et al.,* 2000) |
| *C. perfringens* | NCTC 8237 | 3.18 | 3,002,158 (Shimizu *et al.,* 2002) |
| *E. coli* | n/a | 5.83 | 5,498,450 (Hayashi *et al.,* 2001) |
| *L. monocytogenes* | NCTC 12427 | 3.12 | 2,944,528 (Glaser *et al.,* 2001) |
| *S. aureus* | n/a | 2.86 | 2,700,000 (Mlynarczyk *et al.,* 1998) |
| *S. enterica* | NCTC 13349 | 4.96 | 4,686,000 (Parkhill *et al.,* 2001) |

n/a = not applicable

### 2.10.2 Detection limit calculations for PCR assays

The approximate CFU per PCR was calculated as:

$$\frac{\text{ACC} \times \text{volume of sample extracted} \times \text{amount of eluent used in PCR}}{\text{Elution volume}}$$

The genome copies per PCR were calculated:

$$\frac{\text{DNA concentration} \times \text{volume of DNA per PCR}}{\text{mass of 1 genome.}}$$

### 2.10.3 **Statistical Analysis**

To assess PCR linearity, a standard curve of cycle threshold against CFU or genome copies was produced. The equation of the straight line and correlation coefficient ($R^2$) were calculated using Microsoft Excel. Microsoft Excel was used to plot data regression lines using the 'least squares method' for the standard curves. The $R^2$ value is a comparison of the data regression line and the experimental data points and ranges in value from 0 to 1. A value of 1 indicates that there is no difference between the straight line and the data points. A $R^2$ value of greater than 0.98 indicated that the PCR was linear. The straight line was calculated by Excel using y = mx + b, where the dependant y-value is a function of the independent x-values, m = slope and b = intercept.

The PCR amplification efficiency (*E*) was estimated using the formula $E = 10^{-(1/\text{slope})} - 1$, and expressed as a percentage.

The standard deviations were calculated using the "nonbiased" method and were calculated using Microsoft Excel. Standard deviations were calculated when a mean was determined for more than two replicates. To establish the upper $C_T$ limit for the real-time PCR assay 95 % Confidence level (Cl) was determined. The 95 % Cl was generated by multiplying the standard deviation of the mean $C_T$ by 1.96 and then adding or subtracting this value from the mean $C_T$ to give the upper and lower limits, respectively.

## 2.11 Sequencing reaction methods

### 2.11.1 Purification of PCR amplicons for sequencing reaction

PCR amplicons were purified using a GFX kit (Amersham Bioscience) that contained the columns and wash buffer. A 20 µl aliquot of PCR amplicon was mixed with 500 µl of capture buffer and applied to a GFX column in a collection tube. The column was centrifuged for 30 s at 14,000 *g* and the flow through discarded. The column was washed by adding 500 µl of wash buffer and centrifuged for 30 s at 14,000 *g,* and the flow through discarded. Purified PCR amplicons were eluted by addition of 20 µl of nuclease-free water to the column, incubating for 1 min at room temperature (20 $^{o}$C ± 2) followed by centrifuged at 14,000 g for 30s.

### 2.11.2 Sequencing reaction

Sequencing reaction was performed using a CEQ™ 2000 Dye Terminator Cycle Sequencing (DTCS) quick start kit that was purchased from Beckman Coulter, (High Wycombe, UK). The mastermix, pUC 18 control template, glycogen, sample loading solution and M13 -47 oligonucleotide were supplied in the kit. The sequencing reaction contained 3 µl purified PCR product, 8.0 µl master mix, 2.0 nM of a single oligonucleotide in a 20 µl reaction volume. A positive sequencing control was included that contained 0.5 µl pUC18 control template and was amplified using M13 - 47 oligonucleotides. Cycling conditions consisted 30 cycles of: 96 $^{o}$C for 20 s, 50 $^{o}$C for 20 s followed by 60 $^{o}$C for 4 min. At the end of which a 5 µl aliquot of stop solution (2 volumes of 3 M sodium acetate [Sigma], 2 volumes of 100 mM EDTA [Sigma] and 1 volume of 20 mg ml$^{-1}$ glycogen [Beckman Coulter]) were added to each sequencing reaction prior to ethanol precipitation of the sequencing products.

The entire sequencing reaction product (25 µl) was purified prior to analysis by ethanol precipitation. The DNA was precipitated by the addition of 60 µl ice-cold 95 % (v:v) ethanol and collected by centrifugation at 14,000 *g* at 4 $^{o}$C for 20 min. Pellets were washed twice by adding 200 µl of ice-cold 70 % (v:v)

ethanol and centrifugation at 14,000 *g* at 4 $^{o}$C for 5 min for the first wash, then 3 min for the second wash. The pellet was dried for 30 min and resuspended in 40 µl of sample loading solution. The sequencing PCR amplicons were analysed using a Beckman Coulter CEQ 8000 Genetic Analysis System according to manufacturer's instructions. The amplicons suspended in sample loading solution were transferred to a sample plate and overlaid with mineral oil. The sample plate, buffer plate and polyacrylamide gel cartridge were loaded onto the instrument and analysed using LTFR-1 method.

### 2.11.3 **Analysis of sequencing amplicons**

Chromatograms resulting from the analysis of the PCR amplicons were exported from the Beckman Coulter CEQ 8000. The generated nucleotide sequences were assembled and analyzed using DNAstar (version 5.03; Lasergene Madison, USA). For each target gene, three reads of the sequence were produced by using the forward, reverse and reverse external PCR oligonucleotides. Contiguous sequences were generated from the reads and compared to published sequences (from GenBank) for each gene target using MegaLine (Lasergene).

### 2.11.4 **Basic Local Alignment Search Tool (BLAST)**

Sequences of oligonucleotides and amplicon were analysed *in silico* using Basic Local Alignment Search Tool (BLAST) using the nucleotide database available at:
http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Nucleotides&PROGRAM=blastn&MEGABLAST=on&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome.

## 2.12 Multiplex and Bio-plex assay methods

### 2.12.1 Capture oligonucleotide design for Bio-plex assay

Oligonucleotides complementary to the gene targets (*ceuE, mapA, cpa, cpe, eaeγ, hlyA, nuc, iroB*) were designed following the manufactures guidelines. Capture oligonucleotides were 20 to 21 base in length, with a melting temperature of 55 $^{o}$C (± 1 $^{o}$C) and 40-50 % G:C ratio, while avoiding potential secondary structure or an unstable Δ G. Capture oligonucleotides were synthesised with a 5' terminal amino group with a 12-carbon spacer to allow covalent attachment to the microspheres. Capture oligonucleotides were purified by high performance liquid chromatography and obtained from Sigma Genosys (Haverhill, Suffolk, UK).

### 2.12.2 Carbodiimide coupling of amine-modified oligonucleotides to carboxylated microsphere

Capture probes were bound to microspheres (Luminex Corp. Austin Texas) by carbodiimide coupling of amine-modified oligonucleotides to carboxylated microspheres. To couple the oligonucleotide probes to microspheres 200 pmol of oligonucleotide, 2.5 x $10^6$ microspheres and 25 μg of freshly prepared *N*-(3-dimethylaminopropyl) -*N'*- ethylcarbodiimide (Sigma) were combined in 25 μl of 100 mM 2-(*N*-morpholino) ethanosulfonic acid (MES), pH 4.5 (Sigma); the reaction was incubated at room temperature for 30 minutes. The *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide addition and subsequent incubation were repeated once. After coupling, the microspheres were washed with 0.5ml of 0.02% Tween 20 (Sigma) followed by 0.5ml of 0.1 % sodium dodecyl sulphate. The prepared microspheres were suspended in 50 μl Tris-EDTA (Sigma) pH 8.0 and stored at 4$^{o}$C in the dark until used.

### 2.12.3 **DNA hybridisation to oligonucleotide-coupled microspheres and detection in the Bio-plex suspension array.**

A mixture of each set of oligonucleotide-coupled microspheres was prepared in 1.5 X hybridisation buffer (1.5 M tetramethylammonium chloride [Sigma], 75 mM Tris, pH 8.0 [Ambion], 6 mM EDTA [Gibco], 0.15% Sarkosyl [Sigma]) to approximately 150 microspheres of each set per µl.

In a 96-well flat bottomed plate (Fisher Scientific, Loughborough, UK) 5 µl of PCR product, 33 µl of microsphere mix and 12 µl of 1 x TE was combined. The reaction mix was incubated for 100 $^{o}$C for 5 min, 55 $^{o}$C for 15 min in a thermocycler. The plate was centrifuged at 2,250 *g* for 3 minutes and the supernatant removed. The microspheres were resuspended in 75 µl of detection buffer (*R*-phycoerythrin-conjugated to Streptavidin [Invitrogen] diluted to 4 µgml$^{-1}$ with 1 X hybridisation buffer). Following a 5 minute incubation at 55 $^{o}$C, the samples were analysed in the Bio-plex multiplex suspension array system instrument (Bio-Rad) following the manufacturer's instructions. One hundred and twenty microspheres of each set were analysed which represented 120 replicate measurements. Mean fluorescence intensity (MFI) values were calculated with a digital processor and Bio-plex software. Each assay was run twice and the samples were run in duplicate. The signal to background ratio represents the MFI signals of positive controls verses the background fluorescence of samples containing all components except the amplicon target.

### 2.12.4 **Bio-plex detection of amplicons**

The capture oligonucleotides were hybridised to luminex microspheres (section 2.12.1). Biotinlylated amplicons were produced by amplifying the target pathogens using a biotinylated reverse oligonucleotide in a conventional PCR (section 2.9.3). The working set of capture probes consisting of approximately 150 microspheres of each set. A pool of the biotinylated

amplicons containing 45ng of amplicons from each target gene were hybridised to the working set of capture probes and analysed using the Luminex 100 cytometer instrument following the manufacturer's instructions.

### 2.12.5 **Autodimer software**

Autodimer described by Vallone and Butler (2004) uses an algorithm for determining interstrand complementarily based on the method of Rychlik and Rhoads (1989). Each oligonucleotide sequence of interest was uploaded to the Autodimer program and the software automatically analysed all sequences for interstrand complementarily, oligonucleotide dimerisation and potential for hairpin formation.

### 2.12.6 **Multiplex PCR**

PCR amplification for the detection of target pathogens was performed using puRETaq Ready-To-Go$^{TM}$ PCR Beads (Amersham), 5 µl DNA, and 2.5nM of each forward and biotinlylated reverse oligonucleotide (Table 2.6) in a 25 µl reaction volume. The cycling conditions were 94 $^{o}$C for 2 min, followed by 30 cycles: 94 $^{o}$C for 20 s, 60 $^{o}$C for 20 s followed by 72 $^{o}$C for 30 s. The assay was performed in a T3 Biometra thermocycler.

## 2.13 Field trial methods

### 2.13.1 Sample preparation and culture detection of foodborne pathogens

All enrichment cultures were prepared by the Preston Health Protection Agency (HPA) Collaborating Laboratory or the HPA London Food, Water and Environmental Microbiology Laboratories as part of routine health protection activities and not by this study the methods are full described in the HPA National Methods listed in Table 2.10. A brief overview follows: broths were inoculated with food or environmental samples. Enrichment cultures were prepared by homogenising 25 g food samples with 225 ml of diluent in a stomacher for 1-2 min at high speed (Seward Ltd., UK). The diluents used were: Bolton Broth for *Campylobacter* spp., half-strength Fraser Broth for *Listeria* spp.; Buffered Peptone Water (BPW) for *Salmonella* spp.; modified Tryptone Soy Broth (mTSB) for *E. coli* O157:H7; Cooked Meat Broth for *C. perfringens* and BPW for *S. aureus.* Enrichment cultures were incubated (as detailed below) and then analysed for the presence of *E. coli* O157:H7, *Campylobacter* spp., *C. perfringens L. monocytogenes*, *Salmonella* spp. and *S. aureus*.

For the detection of *Campylobacter* spp. food samples were enriched in modified Bolton Broth for 22 h at 37 $^{o}$C followed by 22 h at 41.5 $^{o}$C. The enrichment culture was then inoculated onto Campylobacter Selective Agar (CCDA), incubated microaerophically 48 h at 37oC, and examined for characteristic colonies.

Food samples were pre-enriched in Cooked Meat Broth for the detection of *C. perfringens*. The inoculated Cooked Meat Broths were incubated for 20-24 h at 37 $^{o}$C. The broths were subcultured to Tryptose Sulphite Cycloserine Agar using a pour plate method and incubated anearobically at 37 $^{o}$C for 20-24 h. After incubation, the agar was examined for typical colonies.

For the detection of *E. coli* O157:H7, foods were pre-enriched in modified Tryptone Soy Broth (mTSB) at 41.5 $^{o}$C for 22 ± 2 h. The cells were isolated

from the broth using immunomagnetic separation (IMS) followed by subculture to cefixime tellurite sorbitol MacConkey Agar (CT-SMAC). After overnight incubation, the Agar was examined for typical colonies.

Half-strength Fraser Broth cultures inoculated with food samples for enrichment of *Listeria* spp. were incubated at 30$^{o}$C for 24 h, followed by transfer of 1 ml to 9 ml of Fraser Broth and incubating at 37$^{o}$C for 48 h. Both the Fraser and Half Fraser enrichment cultures were subcultured onto Listeria Selective Agar (Oxford formulation), after overnight incubation and were examined for the presence of typical colonies.

For the detection of *Salmonella* spp. the food samples were pre-enriched in Buffered Peptone Water (BPW) for 18 h at 37 $^{o}$C. For selective enrichment, 0.1 ml of the BPW enrichment was subcultured in 10ml of Rappaport Vassiliadis Soya Peptone Broth (RVS) and 1 ml of the BPW enrichment was subcultured in 10 ml of Selenite Cystine Broth (SC) or Muller Kauffman Tetrathionate Broth (MKTTn). The RVS and SC broths were incubated for 24 h at 42 and 37$^{o}$C respectively. All broths were subcultured onto Xylose Lysine Desoxychloate Agar (XLD) and modified Brilliant Green Agar (BGA), and examined for typical colonies after overnight incubation.

For the detection of *S. aureus,* food samples were homogenised in BPW to give a ten-fold dilution. A 0.5ml aliquot of the homogenate was inoculated on to Baird-Parker Agar (BPM) and incubated at 37 $^{o}$C for 48 ± 2 h. After incubation, the Agar was examined for typical colonies.

Presumptive colonies of *Clostridia* spp*., Campylobacter* spp., *E. coli* O157:H7, *Listeria* spp., *Salmonella* spp. or *Staphylococcus* spp. were confirmed by standard serological and biochemical tests (Roberts *et al.,* 1995). Enrichment cultures were stored at -20 $^{o}$C until tested by PCR.

### 2.13.2 **Sensitivity of detection of the monoplex assays**

To determine the sensitivity of detection of the monoplex assays the specific organisms. *C. coli* NCTC 12110 and *C. jejuni* NCTC 11180 were cultured in Bolton Broth for 48 h at 37$^{o}$C in microaerophillic conditions. *E. coli* O157:H7 NCTC 12900 was cultured in mTSB Broth for 24 h at 37$^{o}$C. *S. enterica* NCTC 13349 was cultured in BPW for 18 h at 37$^{o}$C. *L. monocytogenes* NCTC 12427 was cultured in half strength Fraser Broth at 30$^{o}$C for 24 h. Serial ten-fold dilutions of the cultures were prepared in their respective un-inoculated broths and ACC performed to determine the colony forming units per ml (CFUml$^{-1}$).

One ml of each serial dilution was subjected to MagNA Pure™ DNA extraction, (section 2.5.1. to 2.5.2), and 5 µl of the resulting DNA amplified in the *ceuE, mapA, eaeγ, iroB* or *hlyA* PCR assay. Each dilution was amplified in four replicates and a standard curve of mean $C_T$ values against CFUml$^{-1}$ was produced. Sensitivity of the duplex assays was performed as described above except a Lenticule disc was added to each of the serial ten-fold dilutions of the cultures prior to DNA extraction. The resulting DNA was then subjected to the ceuE:gfp, mapA:gfp eaeγ:gfp, iroB:gfp or hlyA:gfp PCR assays and a standard curve produced. The probe concentration of the pathogens target was determined by titration from 200 nM to 50 nM per reaction and the concentration that gave the lowest level of detection was chosen.

### 2.13.3 **Oligonucleotide limitation PCR**

A oligonucleotide limitation PCR was performed using standard real-time PCR conditions (section 2.9.7) except for oligonucleotide concentration that were titrated. A matrix of oligonucleotide concentrations for both the *gfp* forward and reverse oligonucleotide was prepared at concentration of 25 nM to 300 nM per PCR reaction. The target DNA was extracted by MagNA Pure™ (section 2.5.1 -2.5.2) from secondary Lenticule discs ($10^4$ CFU per disc).

### 2.13.4 **Method for IPC PCR assays applied to enrichment culture inoculated with naturally-contaminated food samples**

Aliquots of enrichment broth prepared and incubated by the London or Preston FWE laboratories were provided for testing in this assay (section 2.13.1). DNA was extracted from the food homogenates using MagNA Pure™ extraction (section 2.5.1 and 2.5.2). Optimised IPC PCR assays for the detection of *C. perfringens cpe* and *cpa, L. monocytogenes hlyA* and *S. aureus nuc* gene fragments were applied to 5 µl bacterial DNA extracted from enrichment broths (section 2.9.8).

### 2.13.5 **Method for process control PCR assays applied to enrichment culture inoculated with naturally-contaminated food samples**

Aliquots of enrichment broth prepared and incubated by the London or Preston FWE laboratories were provided for testing in this assay (section 2.13.1). To provide a positive control, a single Lenticule disc containing *E. coli* GFP (construction described in Chapter 3) was added to 1ml of enrichment broth culture just prior to DNA extraction. DNA was extracted from the food homogenate using MagNA Pure™ extraction (section 2.5.1 and 2.5.2). Optimised duplex PCR assays ceuE:gfp, mapA:gfp, eaeγ:gfp, hlyA:gfp or iroB:gfp (section 2.9.9) to detect *C. coli, C. jejuni, E. coli* O157:H7, *L. monocytogenes* or *S. enterica* respectively were applied to 5 µl bacterial DNA extracted from enrichment broths.

# CHAPTER 3

## COMPARISON OF DIFFERENT DNA PREPARATION TECHNIQUES TO THE MAGNA PURE™ METHOD FOR PCR DETECTION OF *LISTERIA MONOCYTOGENES* IN FOOD SAMPLES AND INVESTIGATION OF MAGNA PURE™ AS A GENERIC DNA EXTRACTION METHOD APPLIED TO FOODBORNE BACTERIA

### 3.1 Experimental rational

A major limitation in the detection of foodborne pathogens from complex food samples by PCR is the extraction of sufficient intact DNA to perform the assay. Standardized PCR-based methods should include a sample preparation method that concentrates target microorganisms, overcomes the effects of PCR-inhibitory substances and reduces the heterogeneity of biological samples to homogeneous PCR compatible samples (Malorny *et al.,* 2003b). As a consequence, no universal DNA extraction procedure exists (McKillip and Drake, 2004). Liu (2008) reviewed *L. monocytogenes* preparation methods for molecular detection and identification and concluded that further research was needed to optimize sample preparation.

*L. monocytogenes* DNA has been prepared from contaminated food samples using magnetic beads to capture bacterial DNA prior to purification (Nogva *et al.,* 2000), cell lysis by boiling (Oravcova *et al.,* 2007) and commercial products such as the Bacterial Genomic DNA purification kit (O' Grady *et al.,* 2008). Methods that also purify the DNA are advantageous for use in PCR since inhibiting substances are then removed and the DNA is often concentrated. However, these methods are often laborious and can expose the technician to hazardous chemicals.

Automated DNA extraction offers the advantage of DNA purification with reduced technician 'hands on time'. Schuurman *et al.* (2007) compared two automated methods (NucliSENS miniMAG and MagNA Pure™) with a manual silica-guanidinium thiocyanate procedure for sample preparation of *Salmonella* DNA from stool specimens. Findings from this study showed that the automated methods produced a comparable performance to the manual method (Schuurman *et al.,* 2007). An automated DNA preparation method would be advantageous for development of a rapid, high through-put PCR assay for use in the food microbiology laboratory when analysing samples in Listeria food contamination incidents from a diverse range of samples.

In this study, a real-time PCR *L. monocytogenes* assay was selected that was described by Nogva *et al.* (2000). The assay targeted the haemolysin gene (*hlyA*), which is a key virulence factor essential for pathogenicity and is common to all *L. monocytogenes* strains (Vazquez-Boland *et al.,* 2001). It is particularly important that the inhibition of PCR is controlled for in order to prevent the reporting of false negative results (Nogva *et al.,* 2000). To monitor for lack of PCR sensitivity, the real-time PCR assay was duplexed with an internal positive control reaction. The duplex PCR was used to assess how effective DNA preparation techniques were for extracting *L. monocytogenes* DNA.

When a PCR applied to a food sample fails, yet it is known to be contaminated with the target bacteria through culture-based methods, one hypothesis to explain this is that co-extraction of inhibitory components from the food matrix may be inhibiting the PCR reaction. This can be tested using an internal positive control. If a robust nucleic acid preparation technique, that incoperates a method of cell lysis and DNA purification then can the co-extraction of inhibitory components from the food matrix be prevented?

The specific objectives were to:
1) validate a *L. monocytogenes* PCR for use in this study and development of a duplex assay that incorporated and internal positive control to detect false negatives due to inhibition,
2) compare eight DNA preparation techniques to MagNA Pure™ automated DNA extraction method for preparation of *L. monocytogenes* DNA and
3) validate MagNA Pure™ for the extraction of DNA from foodborne bacterial for amplification in real time PCR.
The results sections of this chapter are organised according to work on these objectives.

## 3.2 Results: Validation of *L. monocytogenes* PCR and development of the duplex assay

### 3.2.1 Evaluation of the oligonucleotides and probe concentrations in 5' nuclease real-time PCR for the detection of *L. monocytogenes hlyA* gene

A previously published PCR method was selected for the amplification and detection of the *L. monocytogenes hlyA* gene (Nogva *et al.,* 2000). This assay amplifies a 113-bp fragment of the listeriolysin O gene (*hlyA*). *L. monocytogenes* NCTC 12724 was cultured (section 2.4.1) and DNA extracted by MagNa Pure method (section 2.5.1 and 2.5.2). A ten-fold serial dilution of *L. monocytogenes* DNA was applied to 5' nuclease real-time PCR for the detection of *L. monocytogenes hlyA* gene (section 2.9.7). Amplicons were detected at each cycle of amplification by monitoring the increase in fluorescence of a reporter dye released from the 5' end of the probe by the 5' exonuclease activity of the *Taq* polymerase. The amplification was plotted as $\Delta R_n$, which was calculated from the normalised reporter signal against the number of amplification cycles. The $\Delta R_n$ obtained was approximately one, indicating that the oligonucleotides and probes were at optimal concentrations.

### 3.2.2 Verification of specificity of the PCR assay for the detection of *L. monocytogenes* hlyA gene

A BLAST homology search (section 2.11.4) confirmed that the oligonucleotide and probe sequences were specific for detection of the *hlyA* gene in *L. monocytogenes*. The specificity of the *L. monocytogenes* PCR assay was confirmed using DNA extracted from 20 Gram-positive and Gram-negative bacteria that occur in food and 10 wild-type *L. monocytogenes* strains isolated from food samples. The PCR was found to be 100% specific with amplification detected in DNA extracted from wild-type *L. monocytogenes* strains (mean $C_T$17, SD 0.71), but not from the other 20 bacteria strains tested

or the negative control that consisted of nuclease free water ($C_T \geq 40$) (Table 3.1).

**Table 3.1 Specificity of the 5' nuclease PCR assay for the detection of *L. monocytogenes hlyA* gene applied to bacterial strains.**

| Bacterial strain | NCTC reference | *L. monocytogenes hlyA gene* amplification |
|---|---|---|
| *Aeromonas hydrophila* | NCTC 8049 | Not detected ($C_T \geq 40$) |
| *Bacillus cereus* | NCTC 7464 | Not detected ($C_T \geq 40$) |
| *Bacillus subtilis* | NCTC 10400 | Not detected ($C_T \geq 40$) |
| *Campylobacter coli* | NCTC 206 | Not detected ($C_T \geq 40$) |
| *Campylobacter jejuni* | NCTC 207 | Not detected ($C_T \geq 40$) |
| *Clostridium perfringens* | NCTC 8359 | Not detected ($C_T \geq 40$) |
| *Enterococcus faecalis* | NCTC 775 | Not detected ($C_T \geq 40$) |
| *Escherichia coli* | NCTC 9001 | Not detected ($C_T \geq 40$) |
| *Enterococcus durans* | NCTC 662 | Not detected ($C_T \geq 40$) |
| *Listeria innocua* | NCTC 11288 | Not detected ($C_T \geq 40$) |
| *Listeria ivanovii* | NCTC 11846 | Not detected ($C_T \geq 40$) |
| *Listeria welshimeri* | NCTC 11857 | Not detected ($C_T \geq 40$) |
| *Listeria seeligeri* | NCTC 11856 | Not detected ($C_T \geq 40$) |
| *Proteus rettgeri* | NCTC 7475 | Not detected ($C_T \geq 40$) |
| *Pseudomonas aeruginosa* | NCTC 10662 | Not detected ($C_T \geq 40$) |
| *Salmonella enterica* | NCTC 4840 | Not detected ($C_T \geq 40$) |
| *Staphylococcus aureus* | NCTC 6571 | Not detected ($C_T \geq 40$) |
| *Staphylococcus epidermidis* | NCTC 11047 | Not detected ($C_T \geq 40$) |
| *Vibrio furnissi* | NCTC 11218 | Not detected ($C_T \geq 40$) |
| *Vibrio parahaemolyticus* | NCTC 10885 | Not detected ($C_T \geq 40$) |
| *Listeria monocytogenes* | NCTC 11994 | Detected ($C_T$ 18) |
| *Listeria monocytogenes* | NCTC 12427 | Detected ($C_T$ 17) |
| 10 wild-type *L. monocytogenes* | | Detected (mean $C_T$17 SD 0.71) |
| Negative control | | Not detected ($C_T \geq 40$) |

NCTC = National Collection of Type Cultures; $C_T$ = cycle threshold; SD = standard deviation

### 3.2.3 **Determination of sensitivity of the 5' nuclease PCR assay for the detection of *L. monocytogenes hlyA* gene when applied to whole cell extracted DNA**

To determine the sensitivity of the PCR, an aliquot of *L. monocytogenes* overnight broth culture (section 2.4.1) was extracted by MagNA Pure™ (section 2.5.1 and 2.5.2) and the resulting DNA was ten-fold serially diluted and amplified in the PCR (section 2.9.7). The broth culture was used to determine the ACC (section 2.4.2). The lowest level of detection was 2.25 CFU/PCR, but amplification ($C_T$ 40.71) was only detected in one of the two replicates (Table 3.2).

**Table 3.2 Amplification of whole cell quantified *L. monocytogenes* NCTC 12427 by real-time PCR targeting the *hlyA* gene.**

| CFU/PCR | Cycle threshold ($C_T$) | | |
|---|---|---|---|
| | Replicate 1 | Replicate 2 | Mean (SD) |
| 2.25 | 40.71 | Not detected ($C_T \geq 40$) | 40.71 |
| 22.5 | 36.39 | 36.5 | 36.28 (0.08) |
| 225 | 33.14 | 33.25 | 33.02 (0.08) |
| 2250 | 29.64 | 29.33 | 29.95 (0.22) |
| 22500 | 25.74 | 25.70 | 25.77 (0.02) |
| 225000 | 22.28 | 22.11 | 22.45 (0.12) |
| 2250000 | 18.42 | 18.46 | 18.38 (0.03) |
| Negative control | Not detected ($C_T \geq 40$) | Not detected ($C_T \geq 40$) | |

SD = standard deviation, $C_T$ = cycle threshold.

A standard curve of mean $C_T$ value plotted against CFU/PCR is shown in Figure 3.1. Linear regression was applied to the data points and the equation of the straight line calculated, as shown on the graph. The correlation coefficient ($R^2$) was 0.998, which indicates a high level of confidence in the data with the PCR being linear over a five log dilution range. The slope of the curve (y of -3.58) was used to calculate the PCR amplification efficiency ($E$) using the formula $E = 10^{-(1/\text{slope})} - 1$ resulting in a PCR efficiency of 88 %. The assay

was repeated using a separately prepared overnight culture and the PCR resulted in highly comparable results (y -3.08, $R^2$ 0.994 with a sensitivity of 18 CFU/PCR) indicating that this assay was sensitive and repeatable.

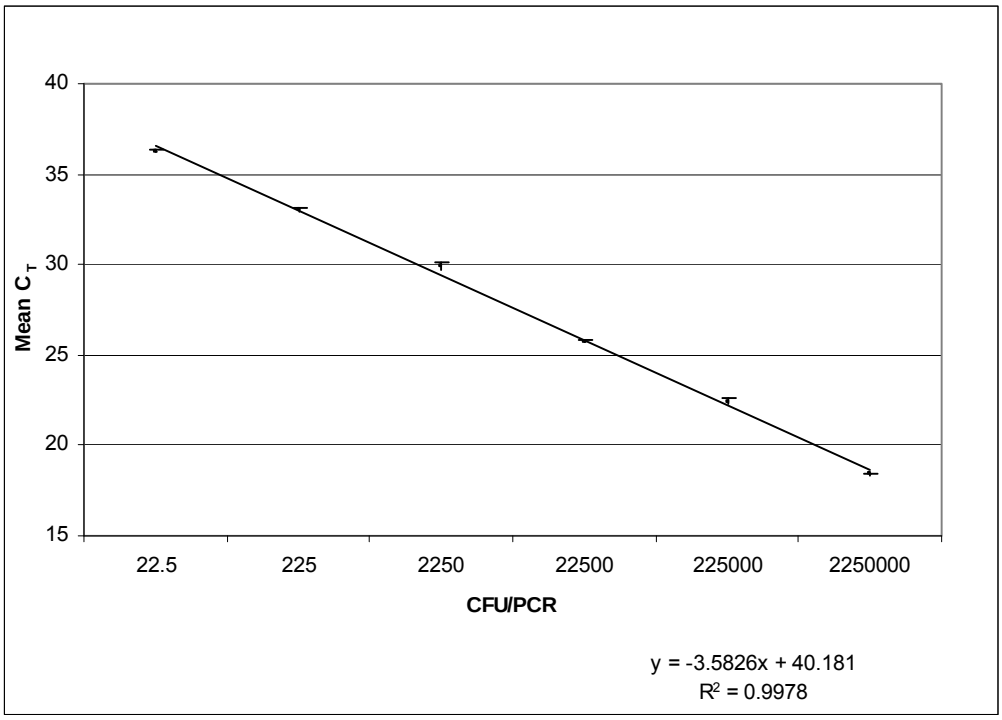



**Figure 3.1 Sensitivity of the *hlyA* real-time PCR using DNA isolated from an overnight culture of *L. monocytogenes* NCTC 12427.**

A ten-fold serial dilution of DNA was amplified using *hlyA* specific oligonucleotide and probe sets. Standard curve plotted using CFU $PCR^{-1}$ against mean $C_T$. The straight lines calculated by linear regression and the data (solid line) yielded a correlation coefficient ($R^2$) = 0.998 and slope of the graph (y) = -3.58. Error bars indicate the standard deviations based on three replicate PCR results.

### 3.2.4 Development of a *L. monocytogenes* internal positive control duplex PCR

The *L. monocytogenes* reaction was duplexed with a commercially prepared internal positive control (IPC) reaction allowing inhibition of the PCR to be monitored *L. monocytogenes* NCTC 12724 overnight broth culture was prepared (section 2.4.1) and DNA was extracted using the MagNA Pure™ (section 2.5.1 and 2.5.2). The resulting DNA was serially diluted in nuclease-free water and applied to the duplex (section 2.9.8) and monoplex PCR (section 2.9.7) assays for detection of *L. monocytogenes hlyA* gene. A standard curve of dilution against $C_T$ was plotted (Figure 3.2). In comparison with the monoplex, the duplex PCR was more sensitive at high concentrations of DNA but sensitivity was reduced at the lower dilutions. The IPC $\Delta R_n$ was considerably higher than the *L. monocytogenes hlyA* gene reaction, which indicated that the PCR favoured the IPC reaction. The duplex was optimised to endeavour to retain the sensitivity of the monoplex.

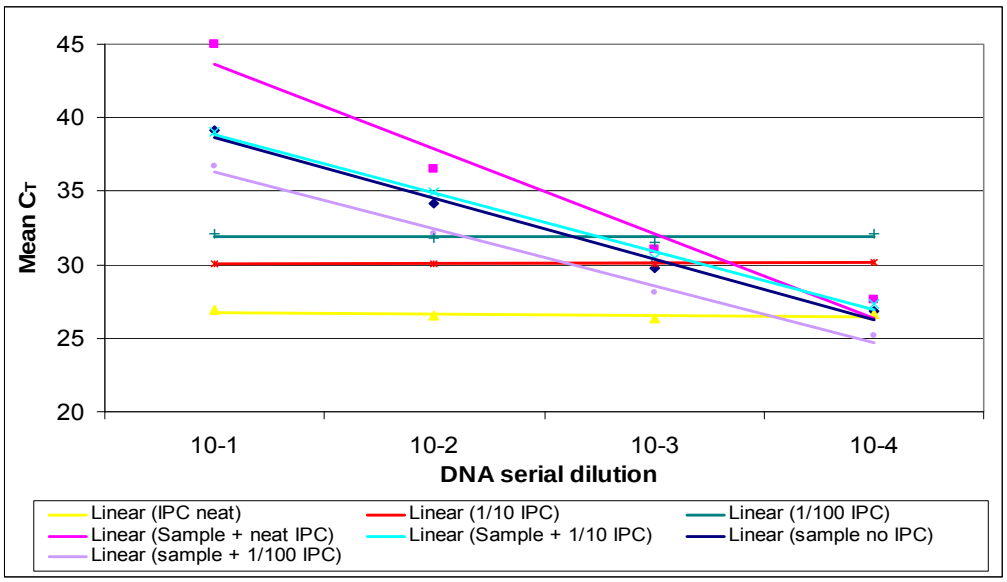



**Figure 3.2 Standard curves generated from *L. monocytogenes* IPC PCR showing the effects of varying the concentration of IPC**

DNA was isolated from an over night culture of *L. monocytogenes* NCTC 12427. A ten-fold serial dilution of DNA was amplified using *hlyA* specific oligonucleotide and probe sets. Standard curve plotted using serial dilution against mean $C_T$. The straight lines were calculated by linear regression. IPC = internal positive control

The kit format of the IPC reaction allowed little scope for optimisation of the duplex reaction as the concentrations of the oligonucleotides, probes and target DNA were not available from the manufacturer's and the oligonucleotides and probes were supplied in a ready-prepared mix. However, the effect of diluting the IPC DNA was investigated. The duplex assay was repeated using: (a) no IPC DNA, (b) IPC at the recommended concentration, (c) a ten-fold dilution and (d) a 100-fold dilution of IPC DNA diluted in nuclease-free water. The results of the PCRs are shown in Table 3.3. *L. monocytogenes hlyA* amplification in the reaction that contained the manufacturer's recommended concentration of IPC DNA resulted in *hlyA* and IPC $\Delta R_n$ 1.41 and 0.91 respectively. In the assay that contained a ten-fold dilution of IPC $\Delta R_n$ 0.89 and 0.93 for the IPC and *hlyA* respectively were obtained. The assay conditions of 100-fold dilution of IPC resulted in a *hlyA* $\Delta R_n$ 0.64 and IPC $\Delta R_n$ 1.22, which were not closely matched. The ten-fold dilution of IPC DNA was selected for subsequent PCR assays given the signal intensity for *hlyA* and IPC was most closely matched.

The IPC reaction was incorporated into the assay to assess if the DNA preparation techniques had affected the sensitivity of the *L. monocytogenes* PCR reaction. The assay was commercially supplied and intended to be a qualitative assay, i.e. either detected or not detected. However, during the validation of the IPC reaction it was noted that a loss of sensitivity could occur in the *hlyA* reaction. To establish loss of sensitivity in the assay the duplex assays were repeated eight times with serial dilutions of *L. monocytogenes* DNA and the mean $C_T$ value of the IPC reaction was calculated. The ten-fold and 100-fold dilution resulted in mean $C_T$ values of 30 and 32 respectively, therefore, a $C_T \geq 33$ was set as the upper limit (Table 3.3). This limit indicated a greater than one log reduction in amplification of the IPC had been lost and it was assumed that the *L. monocytogenes* DNA samples could also have suffered this reduction in sensitivity during amplification.

**Table 3.3 The effect of IPC concentration on the efficiency of the duplex PCR using *L. monocytogenes* NCTC 12427.**

| IPC concentration | IPC $C_T$ (SD) | Amplification in reaction (mean $\Delta R_n$) | |
|---|---|---|---|
| | | IPC | *L. monocytogenes* |
| Neat | 26 (0.338) | 1.41 | 0.91 |
| $10^{-1}$ | 30 (0.228) | 0.89 | 0.93 |
| $10^{-2}$ | 32 (0.244) | 0.64 | 1.22 |

IPC = Internal positive control; $C_T$ = cycle threshold; SD = standard deviation; $\Delta R_n$ = the magnitude of the fluorescent signal.

### 3.2.5 Interpretation of the IPC *L. monocytogenes* duplex PCR

A negative IPC and *hlyA* duplex PCR can be interpreted as negative for detection of *L. monocytogenes hlyA* gene or inhibited due to interference from other molecules in the reaction. In the duplex PCR, if amplification was detected in both reactions, the result is interpreted as positive for the *L. monocytogenes hlyA* gene. If amplification was detected in the IPC reaction but not in the *hlyA* reaction this would indicate a negative result for detection of the *L. monocytogenes hlyA* gene. If no amplification is detected in either reaction component of the PCR, this indicates that there may be inhibition of the PCR reaction. Strong amplification of the pathogen target may reduce the amplification of the IPC. The interpretation of the IPC assay is summarised in Table 3.4.

**Table 3.4 Interpretation of the results generated from duplex internal positive control and real-time PCR assay using *L. monocytogenes* NCTC 12427.**

| Target reaction amplification | | Interpretation of the results |
|---|---|---|
| IPC | *L. monocytogenes hlyA* gene | |
| Detected ($C_T$ <40), | Detected ($C_T$ <40), | Detected ($C_T$ <40), |
| Detected ($C_T$ <40), | Not detected ($C_T$ ≥40), | Not detected ($C_T$ ≥40), |
| [1]Not detected ($C_T$ ≥33), | Detected ($C_T$ <40), | Detected ($C_T$ <40), |
| Not detected ($C_T$ ≥33), | Not detected ($C_T$ ≥40), | Inhibition |

[1]IPC may not be amplified when there is strong amplification of the pathogen target gene.

## 3.3 Results: Comparison of DNA preparation techniques to MagNA Pure™ method

### 3.3.1 Selection of nucleic acid preparation techniques

Seven sample preparation techniques were selected to represent a range of approaches to prepare DNA from *L. monocytogenes* for real-time PCR detection. These included: two simple cell lysis regimes (boiling and *micro*LYSIS®), two cell lysis and inhibitor binding regimes (PrepMan™ Ultra and InstaGene™ matrix) and three complex DNA extraction and purification regimes: the guanadium thiocynate method as described by Boom *et al.*(1990), Bugs' n Beads™ and NucleoSpin® Foods. For each technique the method of cell disruption, nucleic acid purification and presence of a solid support for the DNA are listed in Table 3.5. These techniques were compared to MagNA Pure™ an automated DNA method that is based on the method of Boom *et al*. (1990).

**Table 3.5 Principles of the DNA preparation methods used in this study.**

| Extraction method | Method of cell disruption | Nucleic acid purification | Solid support | Inhibitor binding | Commercial |
|---|---|---|---|---|---|
| Boiling | Heat (100 °C) | None | None | None | No |
| *Micro*LYSIS® | Heat (65 °C), enzymatic lysis | None | None | No | Yes |
| InstaGene™ | Heat (56 °C) | None | None | Yes | Yes |
| PrepMan™ Ultra | Heat (100 °C) | None | None | Yes | Yes |
| Boom | Guanidine isothiocynate, a chaotropic agent that lyses cells and denatures proteins | Two ethanol washes and one acetone wash | Silica suspension in the presence of guanidinium salts | None | No |
| Bugs' n Beads™ | Kit supplied (Power lyse) lysis buffer | Two ethanol washes | Polysaccharide coated magnetic beads bind both bacterial cells and DNA | None | Yes |
| MagNA Pure™ | Heat (65 °C), lysis buffer and Proteinase K, followed by guanidine isothiocynate | Two ethanol washes | Para-magnetic beads bind DNA in the presence of guanidinium salts | None | Yes |
| NucleoSpin® Foods | Heat (65 °C), lysis buffer | Two ethanol washes | DNA bound to a column in the presence of guanidinium salts | None | Yes |

Table ordered by method complexity.

### 3.3.2 **Analytical performance of the DNA preparation techniques assessed by real-time PCR**

The *L. monocytogenes* IPC PCR was used to assess the analytical performance of the seven nucleic acid preparation techniques. *L. monocytogenes* overnight broth culture (section 2.4.1) or spiked milk (section 2.4.5) was ten-fold serially diluted then DNA was extracted by MagNA Pure™ (section 2.5.1 and 2.5.2) from each dilutions. The techniques were applied to a ten-fold serial dilution of *L. monocytogenes* culture and spiked semi-skimmed milk to assess how the methods performed with samples containing high and low concentrations of *L. monocytogenes*. DNA from each dilution was amplified in the *L. monocytogenes* duplex IPC PCR (section 2.9.8). The dilutions were also use to determine the ACC (section 2.4.2).

No amplification $C_T \geq 40$ was detected in the negative controls of MRD or Nutrient Broth for *hlyA* reaction. The positive control of *L. monocytogenes* NCTC 12427 resulted in amplification ($C_T$ 13). These data suggest the suitability of the IPC PCR for detection of *L. monocytogenes* and was utilised to determine the performance of the various DNA extraction methods that are summarised in Table 3.6 and following sections.

**Table 3.6 PCR results from DNA extracted from *L. monocytogenes* NCTC 12427 nutrient broth cultures and spiked semi-skimmed milk using eight different nucleic acid isolation methods.**

| Extraction Method | Pure culture | | | Spiked semi-skimmed milk | | |
|---|---|---|---|---|---|---|
| | PCR sensitivity (CFU $ml^{-1}$) | Inhibition recorded | Sample inhibited | PCR sensitivity (CFU $ml^{-1}$) | Inhibition recorded[1] | Sample inhibited |
| Boiling | $3.04 \times 10^4$ | **No** | None | $3.04 \times 10^4$ | Yes ($C_T$ 33) | $10^{-1}$ dilution only |
| Boom | $5.40 \times 10^4$ | Yes ($C_T$ 37) | Neat sample only | $5.40 \times 10^3$ | **No** | None |
| Bugs & Beads® | $3.05 \times 10^2$ | **No** | None | $3.05 \times 10^5$ | Yes ($C_T$ 33 and $C_T$ 33) | $10^{-3}$ and $10^{-5}$ dilution |
| InstaGene™ | $1.65 \times 10^5$ | Yes ($C_T$ 33) | Neat sample only | $1.65 \times 10^5$ | **No** | None |
| MagNa Pure™ | $3.44 \times 10^3$ | Yes ($C_T$ 35) | Neat sample only | $3.44 \times 10^3$ | **No** | None |
| *micro*LYSIS® | $1.25 \times 10^4$ | Yes ($C_T$ 34) | Neat sample only | $1.25 \times 10^7$ | Yes ($C_T$ >40 and $C_T$ 36) | $10^{-1}$ and $10^{-2}$ dilution |
| NucleoSpin® Foods | $4.52 \times 10^3$ | Yes ($C_T$ 34) | $10^{-3}$ Dilution only | $4.52 \times 10^4$ | **No** | None |
| PrepMan™ Ultra | $6.30 \times 10^6$ | Yes ($C_T$ 34) | Neat sample only | $6.30 \times 10^6$ | **No** | None |

CFU = colony forming units; $C_T$ = cycle threshold, $C_T$> 33 was reported as inhibition detected and $C_T \leq 32$ was reported as inhibition not detected; acceptable or lowest levels of detection values shown in bold (where $R^2$ = >0.98); **Optimal value shown in bold.**

### 3.3.3 **Effect of DNA preparation technique on PCR sensitivity**

The PCR was more sensitive using DNA resulting from the techniques that involved nucleic acid purification (Boom, Bugs' n Beads™, MagNA Pure™ and NucleoSpin® Foods) than the other methods. The two methods that involved magnetic beads as the solid support for the nucleic acid (MagNA Pure™ and Bugs' n Beads™) resulted in the most sensitive detection in the PCR. Pure culture *L. monocytogenes* DNA samples prepared using the Bugs' n Beads™ method resulted in the most sensitive PCR, with the lowest level of detection ($3.05 \times 10^2$ CFU $ml^{-1}$), which was more sensitive then MagNa Pure™ ($3.44 \times 10^3$ CFU $ml^{-1}$).

When the PCR was applied to DNA prepared from spiked semi-skimmed milk, the MagNA Pure™ method resulted in the lowest level of sensitivity of 86 CFU $PCR^{-1}$ compared to the other methods. A 100-fold reduction in PCR sensitivity was observed for DNA samples prepared by *micro*LYSIS® Bugs' n Beads™ and ten-fold for NucleoSpin® Foods method. No reduction in PCR sensitivity was found in samples prepared by MagNA Pure™ or InstaGene™. A ten-fold increase in PCR sensitivity was found with DNA samples prepared by Boom. Nucleic acid prepared by Bugs' n Beads™ had a detection limit of $3.05 \times 10^2$ CFU $ml^{-1}$ and resulted in the most sensitive PCR when applied to culture. Nucleic acid prepared by MagNa Pure™ from spiked semi-skimmed milk resulted in the most sensitive PCR with a detection limit of $3.44 \times 10^3$ CFU $ml^{-1}$.

### 3.3.4 **Effect of DNA preparation technique on PCR inhibition**

Inhibition of PCR reactions can affect reporting of results and requires analysis to ensure assay robustness. The data obtained for the amplification of *L. monocytogenes* and the IPC target are shown in Table 3.6. Inhibition was detected ($C_T$ >33) using the IPC reaction for DNA prepared by all methods except boiling and Bugs' n Beads™ from pure culture. However, PCR

inhibition was only detected with the most concentrated sample (i.e. $10^8$ CFU $ml^{-1}$). In the less concentrated DNA dilutions no inhibition of the PCR was detected from DNA samples prepared using the InstaGene™, PrepMan™ Ultra, Boom, MagNA Pure™ and NucleoSpin® Foods methods.

Following application of the PCR to DNA prepared from the spiked semi-skimmed milk dilution series, inhibition of the *L. monocytogenes* PCR was detected in five DNA samples: one prepared by boiling, three by Bugs' n Beads™ and one by *micro*LYSIS®. Inhibition was detected in the most concentrated sample for these three preparation methods, however in the experiment the sample contained $10^7$ CFU $ml^{-1}$ compared to $10^8$ CFU $ml^{-1}$. The semi-skimmed milk spiked samples ($10^3$ and $10^5$ CFU $ml^{-1}$ dilutions) prepared by the Bugs' n Beads™ method resulted in PCR inhibition. Inhibition seemed to occur randomly throughout the DNA samples prepared by Bugs' n Beads™. The $10^3$ CFU $ml^{-1}$ dilution prepared by the Bugs' n Beads™ method also resulted in no amplification for the *L. monocytogenes* target. However, amplification was detected in the lower $10^2$ CFU$ml^{-1}$ dilution, suggesting the method is not particularly robust. *L. monocytogenes* was detected in all dilutions of spiked semi-skimmed milk, with no inhibition, for the DNA samples prepared by MagNA Pure™.

An unspiked 5 µl aliquot of semi-skimmed milk that had not been subjected to any of the nucleic acid preparation techniques was tested in the *L. monocytogenes* IPC PCR and resulted in $C_T$ 35. This value was recorded as an inhibited PCR, so un-extracted semi-skimmed milk was inhibitory to this PCR. An error of this work was that the experiments comparing the nucleic acid preparation were not repeated to confirm the findings. However, each DNA sample extracted from the spiked milk and culture was tested in duplicate in the PCR.

### 3.3.5 **Cost and ease of use of the DNA preparation methods**

The costs and ease of use of each the nucleic acid preparation methods compared in this study are provided in Table 3.7. The kit-based methods: NucleoSpin® Foods, MagNA Pure™ and Bugs' n Beads™, were considerably more expensive costing between £2.70 and £1.75 per sample. Boom method was the most expensive at £2.84 per sample due to the use of MagNA Lyser beads to break open the bacterial cells. Boiling was the cheapest method, followed by InstaGene™ (37p per sample), *micro*LYSIS® (53p per sample) and PrepMan™ Ultra (83p per sample).

Methods were assessed as to their ease of use based on the number of steps and demand on operator time that is shown in Table 3.7. Most methods were simple to use as they required minimal and simple processing steps, the exceptions were Bugs' n Beads™, NucleoSpin® Foods and Boom. Bugs' n Beads™ method was ranked as moderately difficult due to the manual binding and washing of the magnetic beads, which could easily be lost during the wash steps. NucleoSpin® Foods and Boom were ranked as complex due to the numerous steps that were involved.

**Table 3.7 Cost effectiveness of eight nucleic acid isolation methods investigated in this study.**

| Extraction method | Approximate cost per sample GBP[1] | Ease of use | Approximate 'hands on time' for 20 samples |
|---|---|---|---|
| Boiling | Negligible | Simple | 60 min |
| Boom | 2.84 | Complex | 180 min |
| Bugs' n Beads™ | 1.75 | Moderate | 60 min |
| InstaGene™ matrix | 0.37 | Simple | 90 min |
| MagNA Pure™ | 2.65 | Simple | 60 min |
| *Micro*LYSIS® | 0.53 | Very simple | 10 min |
| NucleoSpin® Foods | 2.70 | Complex | 120 min |
| PrepMan™ Ultra | 0.83 | Simple | 30 min |

[1]Prices calculated on costs of reagents/kit only, and do not include operator time, cost of equipment or general laboratory consumables (ethanol, nuclease-free water, microfuge tubes, gloves, tips and pastettes).

With reference to Table 3.7, the quickest method was *micro*LYSIS® with a 10 minute 'hands on time' followed by PrepMan™ Ultra at 30 minutes. The automated MagNA Pure™ technique had a 'hands on' time of 60 minutes that was comparable to Bugs' n Beads™, and boiling. The least rapid was Boom at 180 minutes, followed by NucleoSpin® Foods at 120 minutes and then InstaGene™ at 90 minutes.

The results of the sensitivity and inhibition to the PCR when it was applied to DNA extracted from *L. monocytogenes* culture and spiked semi-skimmed milk indicated that MagNA Pure™ provides a robust method and for extracting DNA. Therefore, MagNa Pure™ was selected as the method for DNA preparation for the work performed in this study.

### 3.3.6 MagNA Pure™ extraction for the preparation of *L. monocytogenes* DNA from spiked coleslaw and cheese samples

To establish if MagNA Pure™ was applicable to more complex food matrices, the method and PCR was applied to *L. monocytogenes* spiked soft cheese and coleslaw the results are shown in Table 3.8. An *L. monocytogenes* NCTC 12724 overnight broth culture was prepared (section 2.4.1). The *L. monocytogenes* overnight broth culture *L. monocytogenes* was used to spike soft cheese or spiked coleslaw (section 2.4.5). DNA was extracted by MagNA Pure™ (section 2.5.1 and 2.5.2) from ten-fold serial dilutions of the broth culture and spiked foods. The dilutions were also use to determine the ACC (section 2.4.2). DNA from each dilution was amplified in the *L. monocytogenes* duplex IPC PCR (section 2.9.8) and the results are given in Table 3.8.

Previously, DNA extracted using the MagNA Pure™ from pure culture and semi-skimmed milk resulted in a PCR with the lowest level of detection equalling $3.44 \times 10^3$ CFU $ml^{-1}$. This increased one log to $2.5 \times 10^4$ CFU $ml^{-1}$ for DNA extracted from spiked soft cheese and to $2.9 \times 10^4$ CFU $ml^{-1}$ for DNA extracted from spiked coleslaw. No inhibition was detected IPC ($C_T$ 30) in any

of the DNA samples extracted from the spiked food samples or negative controls (maximum recovery diluent and nutrient broth).

**Table 3.8 PCR sensitivity and detection of inhibition for *L. monocytogenes* NCTC 12427 DNA extracted from culture and spiked food samples using the MagNA Pure™.**

| Food matrix or culture | PCR sensitivity as lowest level of detection (CFU $ml^{-1}$) | Inhibition detected |
|---|---|---|
| Coleslaw | $2.9 \times 10^4$ | No |
| Culture (nutrient broth) | $3.44 \times 10^3$ | No |
| Semi-skimmed milk | $3.44 \times 10^3$ | No |
| Soft cheese | $2.5 \times 10^4$ | No |

CFU = colony forming units

## 3.4 Results: Validation of MagNa Pure™ for the extraction of bacterial DNA

The purpose of this experiment was to ascertain whether MagNA Pure™ automated extraction technique could be applied to extract DNA from cultures of the target pathogens: *C. coli, C. jejuni C. perfringens, E. coli* O157:H7*, E. coli* GFP (construction described in Chapter 4), *S. aureus* and *S. enterica*. NCTC cultures of the target pathogens were cultured on Blood Agar (section 2.4.1) then subjected to MagNa Pure™ DNA extraction and the resulting nucleic acid analysed by agarose gel electrophoresis (section 2.5.11). Figure 3.3 shows bands of genomic DNA obtained for *C. jejuni, C. coli, S. enterica, E. coli* O157:H7, *E. coli* GFP*,* and *L. monocytogenes* in lanes 1-6 respectively; however, no DNA band was observed for *C. perfringens* and *S. aureus* (lanes 7-8). The *S. aureus* lane (8) contains a smear that would indicate degraded DNA. Gel bands consistent with 16S and 23S ribosomal DNA are present in samples 1, 4, 6 and 7. Lane 9 is the negative extraction control consisting of nuclease-free water where no DNA or RNA was observed.

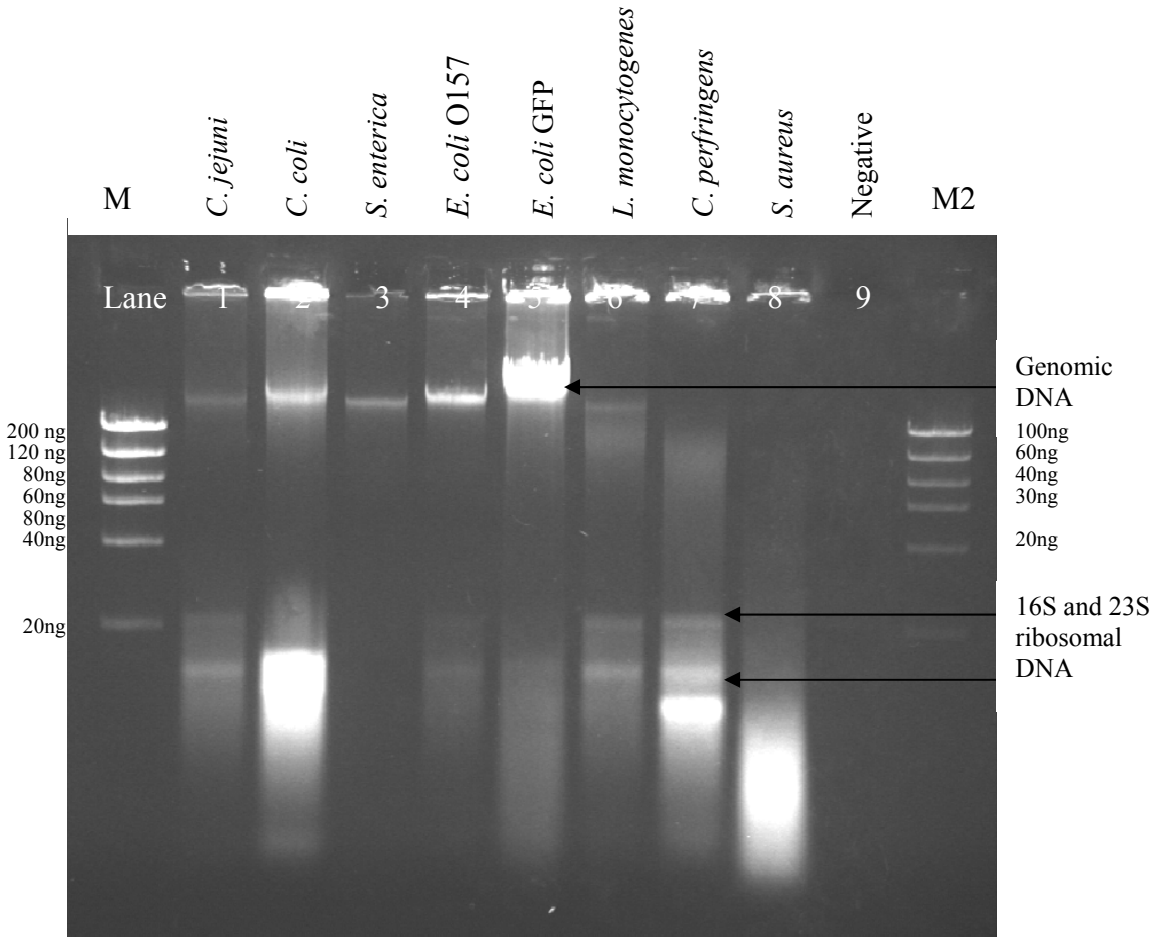



**Figure 3.3 MagNA Pure™ extracted DNA target pathogens: *C. coli, C. jejuni C. perfringens, E. coli, S. aureus* and *S. enterica*.**

DNA analysed on a 0.8% agarose gel. M: High DNA mass ladder (4 µl); M2: High DNA mass ladder (2 µl) 5 µl of extracted material run on 1 x TBE, 0.8% agarose gel. Negative was nuclease-free water for a control for DNA extraction.

### 3.4.1 PCR amplification of DNA extracted by MagNa Pure™ from Foodborne bacteria

The MagNa Pure™ was chosen as a method to prepare DNA prior to PCR amplification. The presence of DNA in MagNa Pure™ nucleic acid preparations had been observed using gel electrophoresis. However, it was essential for this study to establish whether the extracted DNA could be successfully amplified and detected using PCR. At this point in the study, pathogen specific PCR assays were not available for any pathogens except *L. monocytogenes* so a universal bacterial target was required. All bacterial species contain the 16S rDNA gene and it has been exploited as a target for

PCR amplification for identifying bacterial pathogens in clinical samples. A 5'real-time PCR assay for the detection of 16S rDNA was described by Corless *et al.* (2000), however the author reported false positive amplification could occur due to contaminating *E. coli* DNA present in *Taq* polymerase preparations making it unsuitable for this work. However, ethidium monoazide (EMA), a DNA binding dye, can be irreversibly bound to DNA and prevents its amplification in PCR (fully described in Chapter 5). Therefore, an EMA treatment of *Taq* preparation was first investigated to determine if the contaminating DNA in the *Taq* could be rendered unavailable for amplification in PCR and so prevent false positive amplification. Then the 16S rDNA could be investigated to assess if the MagNa Pure™ DNA extraction method could be applied to foodborne bacterial species other than *L. monocytogenes*.

### 3.4.2 **Investigation of EMA cross linking for the removal of contaminating DNA in *Taq* polymerase.**

The EMA cross-linking protocol was applied to the *Taq* polymerase prior to amplification. To ensure no additional bacterial DNA contamination was introduced into the reaction a ten minute UV light treatment was applied to plastic consumables, the PCR buffer, $MgCl_2$ and water. Two samples of *Taq* DNA polymerase (LD Gold *Taq* and Gold *Taq* polymerase) from Applied Biosystems and one sample of *Taq* DNA polymerase from Invitrogen were subjected to the EMA protocol and then used in 16S rDNA PCR, with and without *E. coli* DNA (Table 3.9). No amplification ($C_T$ 40) was observed in the 16S rDNA PCR of the positive control *E. coli* DNA using either of the Applied Biosystems *Taq* DNA preparations. However, amplification was detected with the Invitrogen *Taq* DNA polymerase sample from the *E. coli* positive control sample EMA treated control ($C_T$ 23) and un-treated ($C_T$ 22). There was amplification from the EMA treated nuclease-free water ($C_T$ 34), however this was higher than the non-treated nuclease-free water ($C_T$ 29). Therefore, the EMA had not prevented the PCR assay from proceeding and reduced the false negative signal by 5 cycles.

**Table 3.9 Comparison of brand of *Taq* DNA polymerase on the amplification of false positives in 16S rDNA PCR using *E. coli* NCTC 12900 DNA.**

| Source and brand of *Taq* DNA polymerase | Mean $C_T$ (SD) | | | |
|---|---|---|---|---|
| | EMA treated *Taq* | | Un-treated *Taq* | |
| | water | *E. coli* | water | *E. coli* |
| Applied Biosystems LD Gold | 40 | 40 | 28 | 24 |
| Applied Biosystems Gold | 40 | 40 | 29 | 23 |
| Invitrogen standard | 34 | 23 | 29 | 22 |

EMA = ethidium monoazide; $C_T$ = cycle threshold; SD = standard deviation.

The PCR was repeated with *Taq* preparations treated with dilutions of EMA ranging from 100 to 500 $\mu lml^{-1}$ (Table 3.10). Where *Taq* preparations were treated with concentrations of EMA equal to or above 250 $\mu lml^{-1}$, no amplification was observed in *E. coli* samples, ($C_T > 40$), indicating that the *E. coli* DNA was not detected and the PCR failed. However, *Taq* preparations treated with EMA at 200 $\mu lml^{-1}$ resulted in amplification of the *E. coli* 16S rDNA ($C_T$ 25, SD 0.29).

**Table 3.10 Effect of EMA concentration on the amplification of false positives in 16S rDNA PCR using *E. coli* NCTC 12900 DNA.**

| Concentration of EMA ($\mu lml^{-1}$) | Mean $C_T$ (SD) | |
|---|---|---|
| | EMA treated *Taq* applied to | |
| | water | *E. coli* |
| 150 | 34 (0.65) | 25 (0.32) |
| 200 | 37 (1.33) | 25 (0.29) |
| 250 | 40 | 40 |
| 300 | 40 | 40 |

EMA = ethidium monoazide; $C_T$ = cycle threshold; SD = standard deviation.

Some residual amplification was observed in the EMA treated negative control sample ($C_T$ 37, SD 1.33). A concentration of 200 $\mu lml^{-1}$ EMA was selected as an optimal treatment because the amplification was successful, however $C_T$ for the negative control was $C_T$ 37 therefore only samples in which a $C_T \leq 36$ where considered genuinely positive. Five replicates of each positive and negative control samples were tested and concordant result obtained. The purified *Taq* was then used in 16S rDNA for detection of bacterial DNA after nucleic acid extraction.

### 3.4.3 **Application of the purified *Taq* and 16S rDNA for the detection of target bacterial DNA**

The 16S rDNA PCR using EMA treated *Taq* was applied to MagNa Pure™ extracted DNA from 183 samples from NCTC strains and pure cultures of wild-type bacterial isolates previously identified by HPA reference laboratories. Amplification of the 16S rDNA gene was detected in 151/183 DNA extracts ($C_T$ <36) (Table 3.11). Amplification of *E. coli* DNA was detected (mean $C_T$ 25, SD 0.29) in all five positive control replicates. No amplification was detected in five negative control samples of nuclease-free water ($C_T \geq 36$). In 32 extracts, no or reduced amplification ($C_T = \geq 30$) was observed (Table 3.12). The DNA extracts were prepared from: four *Bacillus* spp. and *Yersinia enterocolitica,* three *Escherichia coli*, two each of *Clostridium butyricum* (NCTC 6084 and 7423), *Shigella boydii, Shigella sonnei* and *Yersinia intermedia,* and single representatives of *Campylobacter sputorum* biovar *faecalis, Clostridium absonum* (NCTC 10984), *Clostridium beijerinckii, Clostridium bifermentans* NCTC 8780), *Clostridium novyi* (NCTC 538), *Clostridium paraputrificum* (NCTC 11833), *Clostridium sporogenes, Enterococcus faecalis, Streptococcus lactis, Providentia sp., Rhodococcus equi, Shigella dysenteriae*, *Shigella flexneri* and *Yersinia frederiksenii*.

**Table 3.11 16S rDNA PCR applied to NCTC and wild-type bacterial strains.**

| Microorganism | No. tested | 16S rDNA PCR result as $C_T$ or mean $C_T$ (SD) |
|---|---|---|
| *Arcobacter butzleri* (NCTC 12481) | 1 | 19 |
| *Arcobacter skirrowii* (NCTC 12713) | 1 | 16 |
| *Campylobacter coli* | 10 | 13 (0.56) |
| *Campylobacter concisus* (NCTC 11485) | 1 | 30 |
| *Campylobacter fetus* subsp. *fetus* (NCTC 10842) | 1 | 16 |
| *Campylobacter fetus* subsp. *veneralis* (NCTC 10354) | 1 | 14 |
| *Campylobacter jejuni* | 10 | 13 (0.63) |
| *Campylobacter lari* (NCTC 11352) | 1 | 17 |
| *Campylobacter mucosalis* (NCTC 11000) | 1 | 17 |
| *Campylobacter upsaliensis* (NCTC 11541) | 1 | 19 |
| *Clostridium perfringens* | 10 | 13 (0.51) |
| *Enterobacter sakazakii* (NCTC 9238) | 1 | 12 |
| *Enterobacter* spp. | 2 | 17 (4.45) |
| *Escherichia coli* (20 WT, NCTC 12900, 9001) | 22 | 14 (0.65) |
| *Hafnia alvei* | 5 | 13 (0.84) |
| *Klebsiella aeruginosa* (NCTC 9528) | 1 | 13 |
| *Listeria innocua* (NCTC 11288) | 1 | 25 |
| *Listeria ivanovii* (NCTC 11846) | 1 | 28 |
| *Listeria monocytogenes* (10 WT, NCTC 11994) | 11 | 14 (0.70) |
| *Listeria welshimeri* (NCTC 11857) | 1 | 28 |
| *Proteus rettgeri* (NCTC 7475) | 1 | 17 |
| *Proteus* sp. | 1 | 13 |
| *Pseudomonas aeruginosa* (NCTC 10662) | 1 | 19 |
| *Salmonella enterica* (10 WT, NCTC 7832) | 11 | 14 (0.66) |
| *Serratia liquefaciens* | 1 | 13 |
| *Shigella boydii* | 5 | 16 (0.62) |
| *Shigella dysenteriae* | 5 | 17 (0.59) |
| *Shigella flexneri* | 5 | 17 (1.23) |
| *Shigella sonnei* | 5 | 16 (0.60) |
| *Staphylococcus aureus* (10 WT, NCTC 1803, 6571) | 12 | 14 (0.59) |
| *Staphylococcus epidermidis* (NCTC 11047) | 1 | 27 |
| *Vibrio furnessii* (NCTC 1218) | 1 | 12 |
| *Vibrio parahaemolyticus* (NCTC 10885) | 1 | 13 |
| *Yersinia bercovieri* | 1 | 11 |
| *Yersinia enterocolitica* (10 WT, NCTC 10460) | 11 | 15 (2.39) |
| *Yersinia frederiksenii* | 1 | 14 |
| *Yersinia intermedia* | 1 | 26 |
| *Yersinia mollaretii* | 1 | 11 |
| *Yersinia rohdei* | 3 | 19 (5.46) |
| Positive control (*E. coli* DNA) | 5 | 25 (0.29) |
| Positive control (nuclease free water) | 5 | 40 |

$C_T$ = cycle threshold; SD = standard deviation; WT = wild-type; NCTC = National Collection of Type Cultures. Pure cultures were cultured overnight on Blood Agar then DNA was extracted using MagNa Pure™ and amplified in 16S rDNA PCR.

**Table 3.12 DNA extracts from wild-type or NCTC cultures subjected to the 16S rDNA PCR which resulted in no or weak amplification.**

| Bacterial strain wild-type or NCTC | Number tested | PCR result as $C_T$ or mean $C_T$ (SD) |
|---|---|---|
| *Bacillus spp.* | 4 | 33(3.5) |
| *Campylobacter sputorum* biovar *fecalis* | 1 | 38 |
| *Clostridium beijerinckii* | 1 | 33 |
| *Clostridium sporogenes* | 1 | 33 |
| *Clostridium absonum* (NCTC 10984) | 1 | 34 |
| *Clostridium bifermentans* (NCTC 8780) | 1 | 35 |
| *Clostridium butyricum* (NCTC 6084) | 1 | 35 |
| *Clostridium butyricum* (NCTC 7423) | 1 | 35 |
| *Clostridium novyi* (NCTC 538*)* | 1 | 35 |
| *Clostridium paraputrificum* (NCTC 11833) | 1 | 34 |
| *Enterococcus faecalis* | 1 | 31 |
| *Escherichia coli* | 3 | 40 (0) |
| *Streptococcus lactis* | 1 | 31 |
| *Providentia sp.* | 1 | 40 |
| *Shigella boydii* | 2 | 37 (3.94) |
| *Shigella dysenterae* | 1 | 36 |
| *Shigella flexnerii* | 1 | 40 |
| *Shigella sonnei* | 2 | 40 (0) |
| *Yersinia enterocolitica* | 4 | 37(3.94) |
| *Yersinia frederiksenii* | 1 | 40 |
| *Yersinia intermedia* | 2 | 40 (0) |
| Positive control (*E. coli* DNA) | 5 | 25 (0.29) |
| Positive control (nuclease free water) | 5 | 40 |

$C_T$ = cycle threshold; SD = standard deviation. Pure cultures were cultured overnight on Blood Agar then DNA was extracted using MagNa Pure™ and amplified in 16S rDNA PCR

## 3.5 Discussion

The aim of the work reported in this chapter was to investigate whether false negative PCR results are associated with the nucleic acid preparation technique. Many existing sample preparation methods for DNA analysis are designed for defined materials such as tissue and bacterial cultures (Rudi *et al.,* 1997). The development of new strategies for nucleic acid isolation and the detection of pathogens in food by PCR has been relatively limited (Rudi *et al.,* 2002). It has been reported that further research is needed to optimize sample preparation for *L. monocytogenes* or molecular detection and identification (Liu, 2008). False negative PCR results may be due to amplification inhibition caused by co-extracted food components (Wilson, 1997). False negative PCR results may therefore result in potentially contaminated material available for human consumption (Nogva *et al.,* 2000). It is therefore essential to use efficient, discriminatory sample processing procedures to turn a heterogeneous biological sample in to a homogeneous PCR compatible sample to enable successful PCR detection (Hoorfar *et al.,* 2004b).

### 3.5.1 Validation of *L. monocytogenes* PCR and development of the duplex assay

Real-time PCR assays have been described for the detection of *L. monocytogenes* in food samples (O' Grady *et al.,* 2008; Oravcova *et al.,* 2007). Detection of *L. monocytogenes* in this study used a previously published 5' nuclease real-time assay described by Nogva *et al.* (2000). The assay was re-evaluated and the efficiency coefficient ($R^2$ 0.998) was comparable to 0.995 found by Nogva *et al.* (2000). The slope of the curve (y = -3.58) is slightly higher than the theoretical optimum (y of –3.32) determined by Higuchi *et al.* (1993), but lower than that found by Nogva *et al.,* (2000) (y = -4.12). The sensitivity of 2.25 CFU/PCR assay with MagNa Pure™ extracted DNA compares favourably to 15 ± 10 CFU/PCR found by Nogva *et al.,* (2000) and the assay was found to be sensitive and specific for the detection of *L. monocytogenes* in this study. The direct correlation of extracted DNA to

quantity of bacterial cells depends greatly on the efficiency and consistency of the method of extraction (James and Genthner, 2008). If the extraction technique is not efficient the quantity of DNA available for amplification may under represent the number of cells (James and Genthner, 2008).

The *L. monocytogenes* PCR was duplexed with a commercially available IPC reaction to form a PCR that could be used to detect *L. monocytogenes* and simultaneously monitor for inhibition of the PCR. The reaction was optimised to ensure the sensitivity of the *L. monocytogenes* reaction was maintained. This assay was then used in the comparison of MagNA Pure™ to seven other nucleic acid extraction techniques for preparing *L. monocytogenes* DNA for PCR detection from contaminated food samples. When applying PCR to DNA extracted from complex matrices, it is essential to monitor PCR inhibition - this can be achieved with an IPC. The use of an IPC gives the operator confidence that negative PCR results are not false-negatives due to inhibition of the reaction.

The inhibition of PCR from food samples was considered due to the heterogeneous nature of these complex matrices. An IPC was successfully used to monitor PCR inhibition to reduce the chance of false negative results. Monitoring inhibition of one extraction technique with an IPC could be used and the extraction technique only modified if there was evidence of PCR failure, rather than modifying the extraction or PCR to suit the specific food sample type. The IPC could be further improved by monitoring the extraction as well as the PCR. This approach would allow one single extraction technique to be used regardless of the food matrix. This would increase confidence that any false negative PCR results would be detected.

### 3.5.2 **Comparison of DNA preparation techniques to MagNA Pure™ method**

*L. monocytogenes* has been implicated in outbreaks of food poisoning associated with contaminated milk, soft cheese and coleslaw (Czajka and Batt,

1994), so were chosen for spiking experiments in this work They contain food components such as protein, fat and calcium ions that have been shown to inhibit both nucleic acid extraction and PCR amplification (Wilson, 1997). Seven sample preparation techniques: boiling, Boom, Bugs' n Beads™, InstaGene™ matrix, *micro*LYSIS®, NucleoSpin® Foods and PrepMan™ Ultra were selected as examples of the different types of DNA preparation techniques currently available.

MagNA Pure™ was successful in that it produced a DNA sample from spiked food that was amplified in a real-time *L. monocytogenes* PCR, without introducing inhibiting compounds. MagNA Pure™ prepared DNA resulted in the most sensitive PCR when applied to semi-skimmed milk and was the second most sensitive when applied to pure broth cultured cells. The highest level of sensitivity in the PCR was 3.05 x $10^2$ CFU $ml^{-1}$, which was attained using Bugs' n Beads™. This level of sensitivity was decreased to 3.44 x $10^3$ CFU $ml^{-1}$ when the methods were applied to spiked milk samples extracted using MagNa Pure™. The sensitivity was decreased a further ten-fold when MagNa Pure™ was applied to soft cheese and coleslaw spiked samples. Schuurman *et al.* (2005) found that in the preparation of viral DNA from clinical samples for detection by PCR, MagNA Pure™ had impaired DNA recovery (up to 14.2-fold) compared to the method described by Boom *et al.* (1990). In contrast, for the samples and PCR targets used in this study, MagNA Pure™ extracted DNA produced better levels of PCR detection than the Boom method. These data concur with the application of MagNA Pure™ extraction for DNA and RNA sample preparation with satisfactory analytical sensitivity in other studies (Fiebelkorn *et al.,* 2002; Kessler *et al.,* 2001; Mohammadi *et al.,* 2003).

The reduction in sensitivity of the assay when applied to complex matrices could be due to the extraction phase, which may have been inhibited by components of the food matrix. The internal control used in this study only monitored PCR inhibition and did not indicate if there was loss of extraction efficiency. Inhibition of the PCR was detected in the DNA samples containing

the highest DNA concentrations prepared by MagNA Pure™, however no inhibition was detected in the more dilute samples that may be due to potential inhibitors or high DNA concentration being diluted. Dilution of a sample has been shown to reduce inhibition (Gregory *et al.,* 2006).

High concentrations of either background flora or target cells can interfere with DNA amplification (Lantz *et al.,* 1998). Dilution of the target DNA stopped the PCR inhibition. If the PCR inhibition was caused by target gene fragments this would not be a concern in a monoplex qualitative assay, however if background flora caused the interference this would effect PCR detection of the pathogen in a food sample that can naturally contain background flora. This could be measured by testing the PCR with high and low concentrations of purely target bacteria with and without the addition of background DNA (sourced from, for example, Salmon sperm).

Fiebelkorn *et al.* (2002) reported that using MagNA Pure™ extracted DNA, they detected no PCR inhibitors and the extraction gave comparable results when compared to manual methods based on lysis of a viral target with chaotropic agent and isopropyl alcohol DNA precipitation. Inhibition of PCR can be reduced by purification of the nucleic acid to remove inhibiting substances (Wilson, 1997) by the use of DNA polymerases that are less sensitive to inhibitors (Abu Al-Soud and Radstrom, 1989) or by the use of amplification facilitators (Hoorfar *et al.,* 2004a). Bovine serum albumin has previously been used as a facilitator to reduce PCR inhibition in faecal and environmental samples (Kreader, 1996). A DNA purification and extraction method coupled with an internal positive control in the PCR was chosen in this study with the intention of producing a generic method that could be applied to any food without any additional sample specific modifications.

MagNa Pure™ was also favoured as a possible generic method as it was automated. Automation of DNA extraction is advantageous in that it reduces human error and variation between operators, thus increasing the precision and reproducibility of results (Kessler *et al.,* 2001). MagNA Pure™ is not the only

automated nucleic acid extraction platform available. The BioRobot 9604 (Qiagen) was compared with the MagNA Pure™ for the preparation of herpes simplex viral DNA in genital and dermal specimens and was reported to be superior to the manual Iso Quick method (Espy *et al.,* 2001). Rantakokko-Jalava and Jalava (2002) suggested that the high cost of the plastic consumables required for MagNA Pure™ extraction procedure can make this method uneconomical if processing less than 32 samples per run, however, it can be a more cost effective approach due to the reduced labour time.

To produce a PCR method for the detection of pathogens in food samples, the method must be rapid, robust, sensitive and cost effective. The sensitivity of a PCR assay is an important criterion for the detection of pathogens in foods (Hill and Keasler, 1991). The *L. monocytogenes* duplex PCR described in this study was robust and rapid with results achieved in less than four hours. However, the PCR combination with any of the investigated extraction techniques would not be sufficiently sensitive to analyse food to comply with the EC Microbiological Criteria of <100 CFU/g *L. monocytogenes* in ready-to-eat (RTE) foods (EC, 2007). Lower detection levels of <10 CFU *L. monocytogenes* per 25g of food have been reported previously (O' Grady *et al.,* 2008; Oravcova *et al.,* 2007). Both studies describe a prior cultural enrichment procedure, which is likely to be required prior to PCR detection in further work to attain a sensitivity that is comparable to the culture method ISO 11290-1.

### 3.5.3 **Validation of MagNa Pure™ for the extraction of bacterial DNA**

Following these initial studies on *L. monocytogenes* reported in Chapter 3, MagNA Pure™ DNA extraction was investigated as a method to prepare DNA from the target bacteria *C. coli, C. jejuni, C. perfringens, E. coli* O157:H7:H7, *E. coli* GFP, *L. monocytogenes*, *S. enterica* and *S. aureus*. DNA was visualised after extraction for all bacteria except *C. perfringens* and *S. aureus.* The lack of visible DNA in the *S. aureus* preparation from the gel electrophoresis may

have been due to the degradation of the DNA by staphylococcal nuclease enzyme in the sample. Cooke *et al.* (2003) modified *E. coli* to express the staphylococcus nuclease and showed if the enzyme is released during cell lysis it acts to hydrolyse host nucleic acid present in the lysate. If sufficient levels of nuclease are produced, complete auto-hydrolysis of the hosts chromosomal DNA to a size non-visible on a 1% agarose gel can be achieved (Cooke *et al.,* 2003). However, in our work the presence of nuclease in the *S. aureus* lysate was not confirmed. To test this hypothesis PrepMan™ Ultra or InstaGene™ matrix extractions (described in Chapter 3) that contain chelating agents that sequester the magnesium and calcium ions and prevent the enzyme from degrading DNA could be investigated for the extraction of *S. aureus* DNA.

The lack of visible *C. perfringens* DNA in the preparation from the gel electrophoresis may have been due to may have been due to insufficient lysis of the cell wall. Rantakokko-Jalava and Jalava (2002) found that a short bead beating step was an efficient method for breaking open cell walls. Therefore, to improve the DNA extraction from *C. perfringens,* the Boom method was investigated as this incorporated a bead beating stage, however genomic DNA was not visualised by gel electrophoresis of the eluent. As the *C. perfringens* DNA was successfully detected by PCR, this anomaly was not investigated any further.

A real-time assay for the detection of 16S rDNA PCR that could be used to detect any eubacteria in a sample described by Corless *et al.* (2000) was investigated in this study for the amplification of bacterial DNA resulting from MagNA Pure™ extractions. However, false positive 16S rDNA PCR reactions resulting from bacterial DNA contamination in reagents have prevented implementation of this PCR (Corless *et al.,* 2000). EMA was used to bind contaminating DNA in *Taq* polymerase preparation preventing its amplification during PCR. The purified *Taq* polymerase preparation was applied to 183 DNA extractions of these 151 had contained DNA that was amplified in 16S rDNA PCR. The extracts were then used for specificity testing for the real-time PCR assays. The positive amplification detected in the

16S rDNA PCR gave confidence that the negative amplification in the pathogens specific PCR assays was due to specificity rather than lack of amplifiable DNA.

When the modified 16S rDNA was applied to the 181 bacterial isolates, the PCR/extraction process failed with 32 samples. The extraction procedure was not repeated and cause for failure not investigated at this time. PCR assays can fail due to technician error however, other samples were extracted and amplified during the same experiment. The process may have failed due to a sample specific problem for example, the cell lysis procedure (heat and guanadinium salt were used here) may have been insufficient for the Gram-negative isolates (*Bacillus* spp. and *Clostridium* spp. containing samples). The aliquots of the eluent resulting from the extraction could have been assayed for the presence of DNA by spectrophotometric analysis or gel electrophoresis. In addition, the PCR should have included an internal positive control to monitor for PCR failure.

Hein *et al.* (2007) described the use of EMA or propidium monoazide (PMA) for eliminating unspecific DNA background in quantitative universal real-time PCR. In contrast to the method used in here, EMA or PMA was added directly to the PCR reaction then exposed to a 650W light. The results were comparable with ones reported here with no detrimental effect on the PCR sensitivity and amplification of unspecific background DNA was eliminated (Hein *et al.,* 2007). The Hein *et al.* (2007) method of adding the EMA to the reaction mix has the advantage of a more simple and rapid procedure that uses less EMA or PMA

Alternative methods of reducing false positive amplification in the 16S rDNA PCR include the treatment of the reagents with DNase I (Carroll *et al.,* 1999), digestion using restriction enzymes or 8-methoxypsoralen combined with UV radiation treatments (Corless *et al.,* 2000). Mohammadi *et al*. (2003) compared digestion of DNA in PCR reagents with *Sau*3AI, ultrafiltration of the PCR reagents and DNAse treatment and found that the restriction enzyme (RE)

digest increased $C_T$ values by three in the negative control sample, without affecting the sensitivity of the assay (Mohammadi *et al.,* 2003). One disadvantage of RE digestion is that this step extends the assay by 50 minutes, in contrast the EMA treatment can be accomplished in 10 minutes. Rueckert and Morgan (2007) applied EMA treatment to conventional block-based PCR and eliminated false positives from 16S rDNA PCR with comparable results to those presented here (Rueckert and Morgan, 2007), however, the author states that this approach is not suitable to real-time PCR due to the complexities of real-time assays. However, our study has shown that EMA treatment can reduce false positive amplification in a real-time 16S rDNA PCR. The rapid and novel real-time EMA approach described here will allow the specific detection of eubacteria 16S DNA templates in samples. The EMA 16S rDNA PCR assay could also be applied to detect bacteria in clinical samples from normally sterile sites, for example, in cerebral spinal fluid.

### 3.6 Conclusion

One hypothesis that was tested in this work was co-extraction of inhibitory components from the food matrix might inhibit the PCR reaction. The use of the IPC indicated that PCR inhibition was detected in DNA samples from spiked milk and from samples that were highly contaminated with the target organism (*L. monocytogenes*). The work also investigated if a robust nucleic acid preparation technique, that incorporates a method of cell lysis and DNA purification (MagNa Pure™ was selected for this work) then can the co-extraction of inhibitory components from the food matrix be prevented. To test the hypothesis the MagNA Pure was applied to *L. monocytogenes* spiked complex foods and was shown to detect the organism without inhibition. This work is the first report of the use of MagNA Pure™ for preparation of *L. monocytogenes* DNA from contaminated food samples for PCR detection. The results indicated that the use of MagNa Pure™, a robust and efficient DNA preparation technique is essential to produce a sample in which the target nucleic acid can be detected by PCR applied to food samples.

The cause of failing to detect the target bacteria in contaminated food samples when using PCR is not limited to inhibition of the reaction by co-extraction of food components. Reasons for PCR failure might be associated the combined sensitivity of PCR and extraction method when applied to large volume of food samples contaminated with low levels of the pathogen, or failure of the DNA extraction. In this study, the PCR combined with MagNA Pure™ had a lower level of detection of $10^3$ to $10^4$ CFU $ml^{-1}$ *L. monocytogenes* in complex food matrices, which was not as sensitive as current culture-based analysis. Initial cultural enrichment, PCR and MagNa Pure™ extraction could return a definitive result within thirty hours, which is a three to seven day improvement on the current culture-based techniques, whilst manitianing culture based levels of sensitivity.

# CHAPTER 4

CONSTRUCTION OF AN *ESCHERICHIA COLI* POSITIVE CONTROL STRAIN EXPRESSING THE GREEN FLUORESCENT PROTEIN (GFP) FOR USE IN PCR DETECTION OF FOODBORNE BACTERIAL PATHOGENS

### 4.1 Experimental rational

The advantages of detecting PCR inhibition by the inclusion of internal positive amplification control (IPC) is recognized for PCR analysis of contaminated food samples. By introducing an internal control into the DNA extraction, this aspect of the assay can also be monitored for inhibition. For example a novel internal control system using a modified plasmid has been described for *B. anthracis* (Panning *et al.,* 2007) and *Coxiella burnetii* (Panning *et al.,* 2008). The plasmid was applied to the lysis buffer prior to extraction and detected by real-time PCR simultaneously with the target organism. If the internal control DNA is incorporated into a surrogate bacteria, i.e. a substitute for the target bacteria, it can be used as a matrix spike and the efficiency of the whole method for that matrix can be determined (Sen *et al.,* 2007).

Bacterial strains chosen as positive controls in laboratory analyses should ideally exhibit characteristics that differentiate them from strains isolated from foods (Noah *et al.,* 2005). Molecular markers or genes conferring specific phenotypes can be used to distinguish, detect and enumerate microorganisms when spiked into a food matrix and then used as positive controls. An example, of a potential gene target is the native green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, which is advantageous as it can be functionally expressed in many organisms and is an ideal reporter for marking bacterial strains (Chalfie *et al.,* 1994). More restriction occurs in marine organisms and so it is not found in most cell types, therefore no background is found.

GFP has been used as a marker for the identification and quantification of several species of Gram-negative bacteria (Unge *et al.,* 1999). Control strains that express GFP have been constructed for use in cultural detection of *E. coli* O157:H7 and *S. enterica* serovar Typhimurium (Noah *et al.,* 2005). The *gfp* gene has also been used a general internal amplification control for real-time PCR detection of *E. coli* O157:H7 and *S. enterica* serovar Typhimurium

(Klerks *et al.,* 2006). Insertion of the *gfp* gene into the chromosome of *Streptococcus gordonii* enabled the strain to be used as a control for cell lysis and real-time PCR (James and Genthner, 2008).

Whilst salmonellae and *E. coli* O157:H7 strains have been used as positive control strains, it is advantageous to choose bacteria that are non-pathogenic for use as control organisms. Strains of *E. coli* have been used as hosts for cloning and expression vectors for specific gene sequences (Chart *et al.,* 2000). The *E. coli* BLR strain is non-pathogenic and a potential host for cloning and expression vectors (Chart *et al.,* 2000) and is also suitable for use as a unique bacterial positive control strain

Bacteria are most commonly labelled with *gfp* by transformation with *gfp*-containing plasmid vectors, however they are not stable unless vector-specific antibiotics are used to ensure a selective pressure to maintain the plasmid (Monday *et al.,* 2003). Incorporation of genes on to the chromosome has the advantages of stable maintenance of the gene in bacterial cells and transfer of the gene to other microorganisms is prevented (Noah *et al.,* 2005). Stable maintenance of *gfp* can be accomplished by integration of the *gfp* gene into the chromosome with mini-transposon cassettes (Noah *et al.,* 2005). The mini-transposon was constructed in plasmid pAG408 (Figure 4.1), which allowed conjugal transfer of the *gfp* gene between compatible strains and insertion of the gene into the chromosome (Suarez *et al.,* 1997).

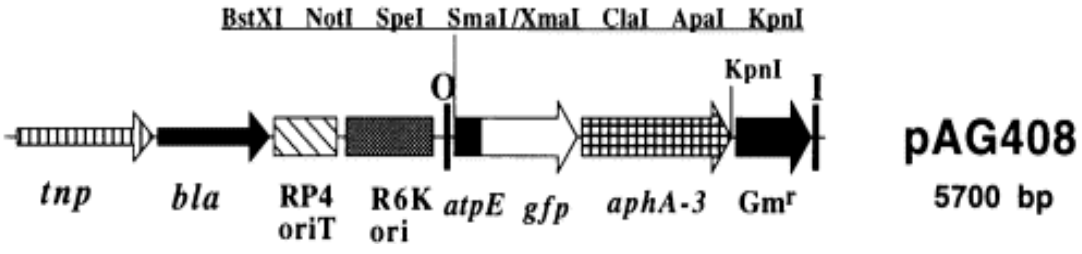



**Figure 4.1 Structure of the mini-transposon pAG408**

Taken from Suarez *et al.* (1997).

*tnp*, Tn*5* transposase; *bla*, *β*-Lactamase encoding gene; RP4 oriT, transfer origin; R6K ori, origin of replication; I and O, inner and outer ends of IS*50*; *BstXI* to *KpnI* restriction endonuclease recognition sites; *atpE,* transcription initiation region; *gfp,* gene encoding green

fluorescent protein; *aphA-3* 3-aminoglycoside phosphotransferase; $Gm^r$, gene encoding gentamycin resistance.

Once constructed, a labelled positive control strain can be added to reactions in a defined amount allowing determination of assay sensitivity. The use of Lenticules, dried discs containing biologically active material in a solid water-soluble matrix (Codd *et al.,* 1998), would enable reproducible controls to be added to each sample. The Lenticule discs provide a means of preserving biologically active material in a quantitative format and have been used successfully as a control material in food microbiology (Codd *et al.,* 1998). Bacteria encapsulated in Lenticule discs have been shown to maintain both bacterial genetic stability (Desai *et al.,* 2006) and viability in stable reproducible numbers (Boyd *et al.,* 2006). Lenticule discs can be used for detection methods where low inoculum is added to a defined quantity of sample and have been used to provide control organisms for UK-wide external quality assessment schemes (Lightfoot *et al.,* 2001).

PCR assays for the detection of bacterial pathogens in contaminated food samples must be sensitive and robust to be comparable to cultural analysis. However, when applied to complex food samples they may lose sensitivity, which leads to the reporting of false-negative results. To attempt to understand this and explain loss of sensitivity either as failure of the PCR, inefficient DNA extraction, interference from components of the food sample, food enrichment broths or large amounts of the target organism, we tested the hypothesis that loss of sensitivity or inhibition may be assessed through the use a known concentration of internal positive control added to the assay at the starting point, prior to the DNA extraction, where the efficiency of the whole process including detection in the complete PCR assay can be monitored.

The specific objectives were to:

1) construct an *E. coli* BLR strain containing the *gfp* gene integrated into the chromosome and

2) to encapsulate the genetically modified strain in a Lenticule disc then evaluate the lenticulated strain as an internal control organism for use real-time PCR applied to food samples.

### 4.2 Results: Construction of a *gfp* expressing strain of *E. coli* BLR.

To generate a positive process control for PCR detection assays of foodborne bacterial pathogens, a GFP-expressing strain of *E. coli* BLR was constructed using the method described by Noah *et al*. (2005). The plasmid pAG408, containing a modified *gfp* gene on a promoter probe mini Tn5 transposon, was isolated from the parental *E. coli* strain CC118 λ pir (section 2.6.1). *E. coli* S17-1 λ pir cells were transformed with pAG408 to generate a donor strain that possessed mobilisation functions, with transformants selected on LB Agar containing kanamycin and streptomycin (section 2.6.2. and 2.6.3). Plasmid DNA was isolated from four transformants, digested with *Kpn*I, and analysed by gel electrophoresis (section 2.6.4). The expected bands were identified, one 1600 bp fragment containing the *gfp* gene, and a 4100 bp fragment consisting of the plasmid backbone (Figure 4.2) from all four transformants indicating that a donor strain had successfully been transformed with pAG408.

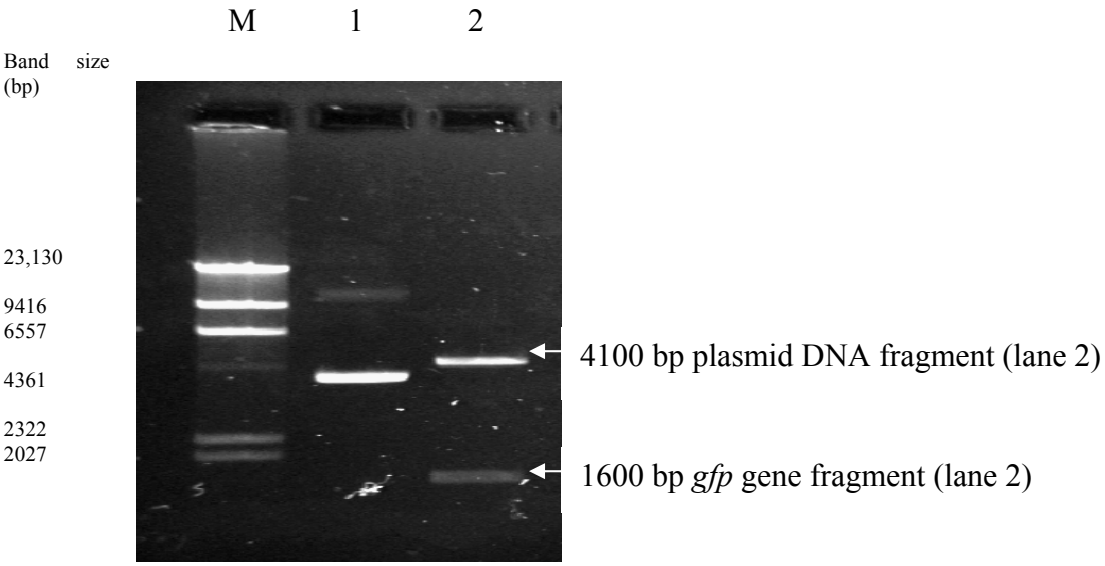
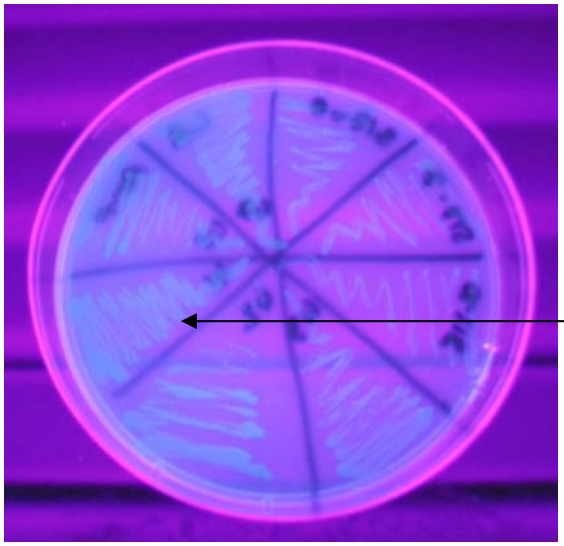



**Figure 4.2 Plasmid pAG408 digested with *Kpn*I showing 4100 bp plasmid DNA fragment and 1600 bp *gfp* gene fragment**.

Lane M, *HindIII* λ DNA marker; Lane 1, uncut pAG408 plasmid; Lane 2, pAG408 digested with *KpnI* containing 1600 bp *gfp* gene fragment and 4100 bp fragment of plasmid DNA pAG408 run on a 0.8% agarose gel.

One *E. coli* S17-1 λ *pir* pAG408 transformant was selected and filter-mated with *E. coli* BLR (section 2.6.5). The *E. coli* S17-1 λ *pir* pAG408 donor cells were not selected against, because GFP is not expressed in this strain, as there is no promoter to drive *gfp* expression. The *E. coli* BLR parent was selected against using kanamycin. The resulting transconjugates (approximately $10^8$ CFU $ml^{-1}$) containing pAG408 were selected with kanamycin (marker on plasmid) and GFP expression was detected by presence of fluorescent colonies under UV light.

One hundred fluorescent colonies were replica-plated on to agar plates containing kanamycin or ampicillin. Loss of the pAG408 in the transconjugates was confirmed by sensitivity to ampicillin. Successful integration of the transposon containing the promoterless *gfp* gene in the recipient and its expression from native promoters in eight isolates was confirmed by visualisation under UV light (Figure 4.3).



Fluorescence of the green fluorescent protein expressed by *E. coli* BLR transconjugates in the presence of UV light.

**Figure 4.3 Fluorescence of the green fluorescent protein expressed by *E. coli* BLR transconjugates in the presence of UV light.**

Five kanamycin resistant (integration of mini-transposon encoding *aphA* from pAG408), ampicillin sensitive (loss of parental vector) colonies were selected. DNA was extracted from the colonies (section 2.5.8) and the presence of the

*gfp* gene confirmed by the detection of a 122 bp *gfp* gene fragment by PCR (section 2.9.3). A single transconjugate exhibiting the strongest fluorescence was then selected as the *E. coli* GFP strain, and the presence of the *gfp* gene confirmed by conventional PCR as described previously (Figure 4.4). The intensity is likely to be dependant upon the integration site within the genome and proximity of strongly expressing promoters. Stability of the *gfp* gene in the *E. coli* GFP strain was confirmed by successive sub-culture of the strain over three months with detection the *gfp* gene following each sub-culture by conventional PCR. The stability of GFP expression was further confirmed after subculture by visualisation of fluorescence in the presence of UV light.

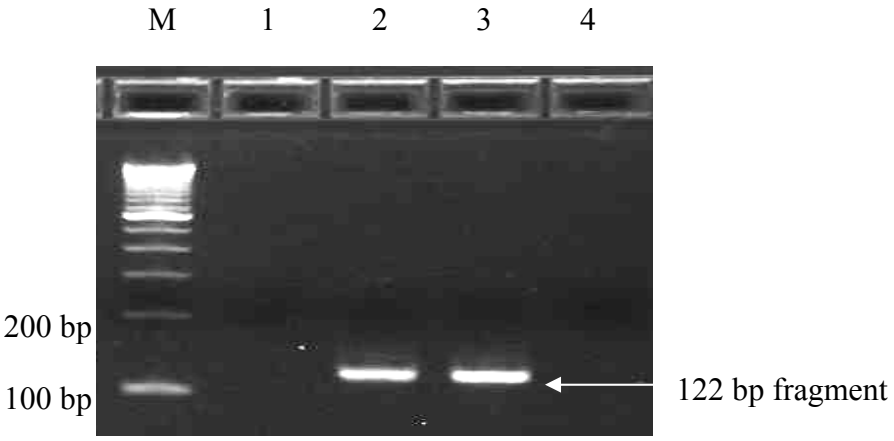



**Figure 4.4 PCR amplification of 122bp fragment of *gfp* gene from *E. coli* GFP.**

Lane M 100bp ladder; Lane 1 *E. coli* BLR; Lane 2 *E. coli* GFP; Lane 3 *E. coli* S17- λ pir pAG408 (positive control); Lane 4; negative control run on a 4 % agarose gel.

### 4.2.1 **Development and evaluation of 5' nuclease real-time PCR for the detection of the *gfp* gene**

A 5' nuclease real-time PCR assay for detection of a 77 bp fragment of the *gfp* gene was designed using Applied Biosystems Primer Express software version two (section 2.5.9). A BLASTN homology search (section 2.11.4) confirmed that the oligonucleotides and probe sequences were only homologous to the *gfp* gene (sequence based on Genbank accession number M62654) contained in the *E. coli* BLR-GFP. The specificity of the *gfp* real-time PCR was confirmed using 160 DNA extracts from bacterial, viral (cDNA) and parasitic sources (organisms listed in Tables 2.1 and 2.2). Each bacteria was cultured (section 2.4.1) then DNA was extracted using MagNa Pure™. The DNA was applied to the *gfp* real-time PCR (section 2.9.5) using gfpR and gfpR primers (Table 2.6). The PCR was found to be 100% specific with no amplification detected ($C_T \geq 40$) from any bacterial, viral or parasitic sources.

To determine the sensitivity of the PCR, an aliquot of plasmid DNA from the parent strain *E. coli* CC118 $\lambda$ pir (ATCC 87653) was diluted ten-fold in nuclease-free water and amplified in the real time *gfp* PCR (section 2.9.5). The lowest level of detection was estimated to be 10 copies of the gene. The $R_n$ for the gfp real-time PCR assay was 1.0, which indicated that the oligonucleotides and probe concentrations were optimal. The assay was 100% efficient (y = -3.288), linear ($R^2$ = 0.999) over an eight log dilution series and the 95% confidence levels did not overlap (p = <0.05) (Figure 4.5). A one log difference in amplification resulted in a $C_T$ change of 3.2 cycles and indicated that the assay provides an accurate quantification over an eight log dilution series, which was maintained to the lowest level of detection (10 gene copies).

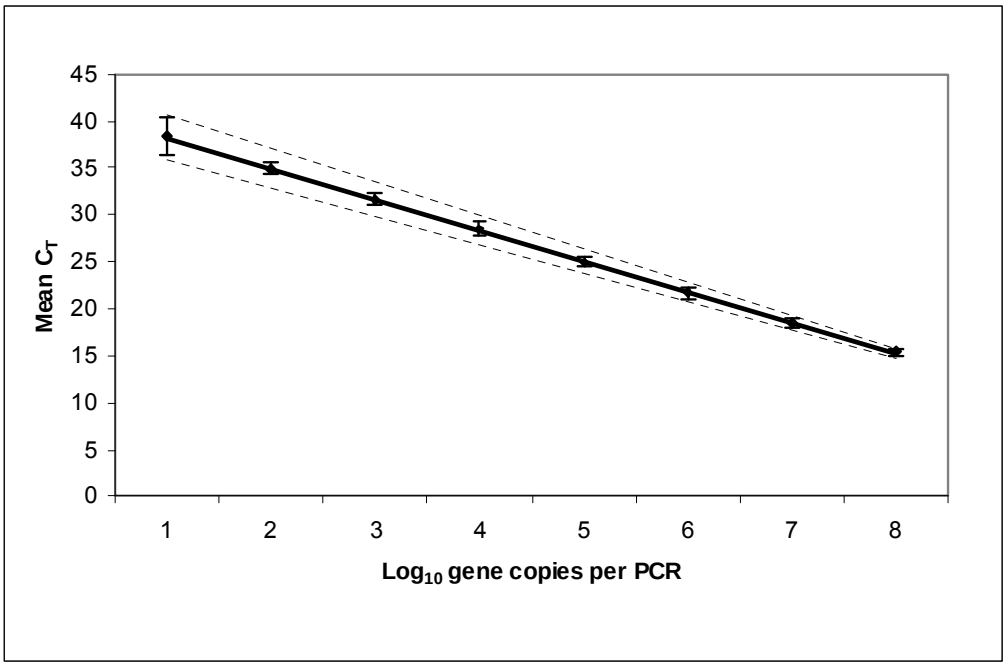



**Figure 4.5 Sensitivity of the gfp real-time PCR using DNA isolated from pAG408 plasmid.**

Quantified ten-fold serial dilutions amplified using gfp specific oligonucleotide and probe sets. Standard curve plotted using $Log_{10}$ copies of *gfp* gene against mean cycle threshold ($C_T$). The straight lines calculated by linear regression and the data (solid line) yielded a correlation coefficient ($R^2$) = 0.999 and slope of the graph (y) = -3.288. Upper and lower 95% confidence intervals shown as broken lines. Error bars indicate the standard deviations based on four replicate PCR results.

### 4.2.2 Detection of *E. coli* GFP in spiked semi-skimmed milk

The *gfp* PCR was applied to the simple matrices of MRD and semi-skimmed milk. The real-time PCR assay for the *gfp* gene (section 2.9.5) was applied to MagNa Pure™ DNA extracted (section 2.5.2 and 2.5.3) from a ten-fold dilution series of *E. coli* GFP in both MRD and spiked semi-skimmed milk. Less than three-cycle difference was detected in the $C_T$ values obtained using DNA from MRD or semi-skimmed milk samples in all samples except the $10^6$ CFU $ml^{-1}$ samples of spiked semi-skimmed milk (Table 4.1). The lower limit of detection was estimated to be 10 CFU $ml^{-1}$ when the assay was applied to either DNA extracted from MRD or semi-skimmed milk samples, indicating that it was a sensitive and robust assay.

**Table 4.1 Amplification of the *gfp* gene from *E. coli* GFP DNA extracted from a decimal dilution of spiked semi-skimmed milk or broth culture.**

| CFUml$^{-1}$ of *E. coli* GFP | PCR detection of *gfp* as mean $C_T$ (SD) | |
|---|---|---|
| | Semi-skimmed milk | Broth culture |
| $10^6$ | 22 (0.18) | 17 (0.50) |
| $10^5$ | 23 (0.27) | 22 (0.32) |
| $10^4$ | 27 (0.13) | 26 (0.17) |
| $10^3$ | 29 (0.41) | 30 (0.30) |
| $10^2$ | 35 (0.37) | 34 (0.46) |
| $10^1$ | 38 (0.30) | 37 (0.88) |
| 1 | >40 (0) | >40 (0) |
| Negative control | >40 | >40 |

CFU = colony forming units; *gfp* = green fluorescent protein gene; $C_T$ = cycle threshold; SD = Standard deviation.

## 4.3 Results: Development of a Lenticule encased *E. coli* GFP strain for use as an internal positive control organism

### 4.3.1 Lenticulation of the *E. coli* GFP

Primary LENTICULE discs containing *E. coli* GFP were produced (section 2.6.6). Five discs from the batch were dissolved in Page's saline and an ACC indicated (section 2.4.1) that each disc contained approximately $10^8$ CFU. The presence of the *gfp* gene in the Lenticule was confirmed by extracting the DNA (section 2.5.8) from a dissolved discs and amplification the *gfp* gene by conventional PCR (section 2.9.3).

Amplification of *gfp* gene from whole cell and a lenticule encapsulated *E. coli* GFP was compared. The monoplex real time PCR assay (section 2.9.7) for the *gfp* gene was applied to DNA extracted (section 2.5.8) from a 10-fold dilution series of an overnight culture of *E. coli* GFP (section 2.4.1) and a reconstituted *E. coli* GFP lenticule disc. No difference was detected in the lowest level of sensitivity of the PCR assay for the overnight culture and lenticulated *E. coli* GFP. The lowest level of detection was found to be equivalent to 16 CFU ml$^{-1}$

or eight CFU per PCR, indicating a very sensitive PCR assay. GFP was fully expressed by >99% of colonies after lenticulation, and *gfp* amplicons were detected by real-time or conventional PCR with no difference in $C_T$ value from the real-time PCR after 12 weeks storage.

### 4.3.2 Optimisation of the lenticulated *E. coli* GFP as a positive process control

The use of *E. coli* GFP Lenticule discs as a process control for PCR detection assays was investigated. A primary *E. coli* GFP Lenticule disc ($10^8$ CFU per disc) was spiked into the broths used for food sample enrichment. The broths tested included BPW for the enrichment of *Salmonella* spp., Half Fraser Broth for the enrichment *Listeria* spp. and Bolton Broth for the enrichment of *Campylobacter* spp. MagNA Pure™ extracted DNA (section 2.5.2 and 2.5.3) was amplified by *gfp* real-time PCR (section 2.9.7) from all broths resulted in a $C_T$ value of 17 for the *gfp* gene.

The experiment was repeated using an overnight broth culture of each NCTC strain of target pathogen grown in the appropriate broth: *S. enterica* NCTC 13349 (BPW), *L. monocytogenes* NCTC 12427 (Half Fraser Broth), *C. jejuni* NCTC 11168 (Bolton Broth) and *C. coli* NCTC 12110 (Bolton Broth). Amplification of the *gfp* gene ($C_T$ 17) was detected from DNA extracted from all the inoculated broths and this result was concordant with the un-inoculated broths. PCR testing was performed in duplicate for each DNA sample extracted from the broths, with concordant results obtained. This experiment indicated that the *gfp* target could be added to broth cultures with or without target bacteria and be detected without affecting the sensitivity of the *gfp* PCR reaction.

To quantify the loss of PCR sensitivity, it was necessary to add a known concentration of internal control to the PCR. A low concentration of process control was required to prevent competition for reagents between the process control and target reaction that may result in loss of sensitivity for the detection of the target DNA by PCR. The primary Lenticule discs contained

$10^8$ CFU per disc. At this high concentration it may cause loss of PCR sensitivity when the assay is duplexed pathogen assay (as described in Chapter 3). Therefore a lower level of control target that could be added to the PCR and yet maintain a stable $C_T$ was investigated.

A primary Lenticule disc was resuspended in Pages saline and *E. coli* GFP concentration was re-confirmed by ACC as 1.6 x $10^8$ CFU per Lenticule disc. A serial ten-fold dilution was tested in triplicate in the real-time *gfp* PCR. A stable $C_T$ was detected up to the dilution $10^2$ CFU $ml^{-1}$ with the *gfp* gene detected in all the dilutions. At $10^1$ CFU $ml^{-1}$ *E. coli* GFP, the *gfp* gene was detected in two of the three triplicates that caused the increase in standard deviation (Table 4.2).

**Table 4.2 Amplification of the *gfp* gene from *E. coli* GFP DNA extracted from a decimal dilution of a $10^8$ CFU Lenticule disc.**

| CFU $ml^{-1}$ *E. coli* GFP | CFU/PCR | PCR detection of *gfp* in mean $C_T$ (SD) |
|---|---|---|
| $10^8$ | 5000000 | 15 (0.35) |
| $10^7$ | 500000 | 18 (0.22) |
| $10^6$ | 50000 | 21 (0.39) |
| $10^5$ | 5000 | 24 (0.38) |
| $10^4$ | 500 | 29 (0.30) |
| $10^3$ | 50 | 32 (0.31) |
| $10^2$ | 5 | 36 (0.53) |
| $10^1$ | 0.5 | 39 (1.26) |
| 1 | 0.05 | >40 |
| Negative control | | >40 |

CFU = colony forming units; *gfp* = green fluorescent protein gene; $C_T$ = cycle threshold; SD = Standard deviation.

Based on these results, the Lenticule discs would have been produced at $10^2$ CFU per disc, however the secondary Lenticule discs were produced at $10^4$ CFU per disc. At $10^4$ CFU per Lenticule disc, the internal control was present at approximately 500 CFU per PCR following DNA extraction assuming 100% extraction efficiency. The $10^4$ CFU $ml^{-1}$ *E. coli* GFP dilution was equal

to approximately 500 CFU per PCR and resulted in an amplification $C_T$ of 29 (SD 0.30). By increasing the level of control target in the Lenticule disc, it was also possible to measure loss of sensitivity in the assay. The $C_T$ value resulting from the next ten-fold dilution ($10^3$ CFU $ml^{-1}$) of *E. coli* GFP $C_T$ value was 32, which was defined as the 'loss of sensitivity' threshold, this value would indicate a ten-fold loss of sensitivity.

To ensure that the lenticulation did not affect the stability of the GFP mini-transposon in the strain, expression of GFP was checked by culture and visualisation under UV illumination. GFP was expressed by >99 % of colonies following lenticulation and no difference were observed in amplification of the *gfp* gene by real-time PCR after 12 weeks storage. The robustness of the Lenticules was confirmed by amplifying the *gfp* gene extracted from 10 Lenticule discs in the real-time *gfp* PCR with the mean $C_T$ equalling 28.9 (SD 0.723). To validate this assay fully for use in the diagnostic laboratory, it required optimisation in duplex PCR assays with the target pathogens, which is described fully in Chapter 6.

### 4.4 Discussion

The aim of the work reported in this chapter was to investigate whether an internal control organism could be developed that could be used to monitor failure in both the DNA preparation and PCR, to prevent false negative interpretation of PCR results. Detection of pathogens in food samples by PCR requires the use of adequate controls that ensure the quality of all assays and processes used. It is particularly important that inhibition of PCR be controlled to prevent the reporting of false negative results. In the proposed ISO standards for PCR-based detection of pathogens in food, the use of controls including an internal control that specifically controls for PCR inhibition is mandatory (Anon., 2002). The increasing and widespread use of standardised protocols, reagents and control material has considerable advantages of reproducibility between analysts and laboratories. However, it has been recognised that there is a lack of suitable and validated control material

available for use with molecular tests applied for the detection of bacterial pathogens in food samples (Hoorfar *et al.,* 2004b; Malorny *et al.,* 2003b). This problem has been addressed in this chapter through the development and evaluation of a standardised, validated internal control that monitors PCR inhibition in detection assays for foodborne pathogens using an *E. coli* strain marked with a *gfp* gene.

### 4.4.1 **Construction of the *E. coli* GFP strain**

The process control described here is based on a foreign target sequence (the jellyfish green fluorescent protein gene) that is unlikely to occur in the original sample. The control was produced as a Lenticule disc, so allowing the strain to be easily introduced into the assay procedure in a stable quantified format. The *E. coli* BLR strain was selected for construction of a GFP-expressing bacterial strain as it is non-pathogenic and suitable as a host for cloning and expression vectors (Chart *et al.,* 2000). An integrated transposon-based copy of *gfp* was selected because it has been shown that plasmid copy number varies throughout the bacterial growth cycle (Turgeon *et al.,* 2008). A single integrated copy therefore affords increased reproducibility within the assay.

The *E. coli* GFP strain constructed in this work was not strongly fluorescent under UV light. This would make it difficult to differentiate from food isolates if phenotypic characteristics were solely used for its identification, for example, as an internal control in culture-based analyses. This may have been due to control of the expression of the protein being driven from a weak promoter. The mini-Tn5 transposon method used to construct the strain is such that the position of gene incorporation into the chromosome is semi-random (de Lorenzo *et al.,* 1993; Suarez *et al.,* 1997), and so the promoter is currently unknown. The position of the gene could have been identified using ligation mediated PCR, a cloning approach or southern blot analysis; however since the strain worked effectively as an extraction and PCR control and phenotypic identification of the strain through detection of the green fluorescent protein was available, knowledge of the transposon insertion site was not required.

### 4.4.2 **Lenticule encased *E. coli* GFP strain used as an internal positive control organism**

Primary Lenticule discs contained $10^8$ CFU per disc; however, this was reduced to $10^4$ CFU per disc in the secondary discs. By adding a reduced amount of control material to the reaction, competition for reagents between the control and the pathogen target reaction can be reduced, favouring the pathogen instead of the control target. A reduction in the sensitivity of the different targets in multiplex reactions can be due to competition for reagents (Klerks *et al.,* 2004). It has been recommended that between 50 and 500 copies per PCR of internal control is optimal for internal control detection without causing competition with the target amplicon (Ballagi-Pordany and Belak, 1996). At a concentration of $10^4$ CFU per Lenticule disc, the internal control will be present at approximately 500 CFU per PCR following DNA extraction assuming 100% extraction efficiency. A concentration of $10^4$ CFU per Lenticule disc may be too high when compared to the recommendations described by Ballagi-Pordany and Belak (1996). However, if the control is present at a lower concentration, it may be over sensitive to small variations encountered during testing, for example, from analyst to analyst or from batch to batch of reagents. The optimised process control was successfully detected by the real-time PCR from enrichment broths or semi-skimmed milk samples spiked with NCTC cultures demonstrating its utility and robustness.

The results of the amplification from the Lenticule disc (section 4.3.5) suggest that the assay is capable of a positive amplification ($C_T$ 39) from 0.5 CFU per PCR. This was an averaged (mean) result in which the *gfp* gene was detected in two of three replicates. Although the amount of target DNA was not measured in the Lenticules, an approximation could be made with reference to the standard curve produced for the *gfp* assay (Figure 4.5) where a $C_T$ 39 is approximately equal to 10 copies of the gene. It was assumed that the gene was present as a single copy on the *E. coli* GFP genome. Therefore, the result from the Lenticule and standard curve were not concordant. However, the

Lenticules are likely to contain more target genes than expected because the Lenticules also contained DNA from dead organisms present in the overnight broth culture.

Although the quantification of target, whether measured in CFU or genome copies per PCR, was important for this experiment, obtaining the same consistent $C_T$ value from Lenticule disc to disc in a batch was considered more important for the purpose of this work. The purpose of the Lenticule discs was for measuring assay inhibition or loss of sensitivity. The Lenticules were shown to produce concordant $C_T$ values from separate amplifications from 10 Lenticule discs indicating that they were a reliable and reproducible control.

For the remainder of this study the sensitivity of 'PCR assays' (i.e. including extraction) was measured in CFU $ml^{-1}$ per PCR. The PCR sensitivity based solely on the performance of the PCR when challenged with purified and quantified DNA was measured in genome copies. Measuring assay sensitivity (in CFU per PCR or ml) and PCR sensitivity (in genome copies) enabled the results to be as accurate as possible.

The method of constructing the *E. coli* GFP produced in this study followed the method by Noah *et al.* (2005), who applied the method to modify an *E. coli* O157:H7 strain and a *S. enterica* Typhimurium to express the GFP. The modified organisms where then used as positive control strains for specific bacterial culture detection methods. Sen *et al.* (2007) also produced a modified *E. coli* internal control organism for use during real-time PCR detection of *Helicobacter pylori* in water samples. The authors report the importance of including internal controls when designing assays to enable them to be robust and accurate screening tools (Sen *et al.,* 2007).

## 4.5 Conclusion

A real-time PCR was developed for the detection of a green fluorescent protein gene present in a genetically modified *E. coli* strain. A predetermined quantity ($10^4$ CFU per disc) of the strain was encapsulated in a Lenticule disc and was shown to be a suitable internal positive process control for 5'nuclease PCR assays. This control may provide an improved method for monitoring loss of assay sensitivity and PCR inhibition. This will prevent false negative interpretation of results, which is highly applicable to high-throughput and routine diagnosis of pathogens in complex food matrices. The *gfp* gene was successfully detected from secondary Lenticule discs spiked into culture broths used for the enrichment of *Salmonella, Listeria* spp. and *Campylobacter* spp. with competing target organisms. In this study, the *E. coli* GFP control organism was produced for PCR detection methods rather than culture. *E. coli* GFP control organism and the gfp PCR can be used with alternative target bacterial pathogen that are detected using standard 'TaqMan' real-time PCR. This control offers the advantage that it can be duplexed with specific pathogen identification 5'nuclease real-time PCR assays (Chapter 6) and can be used as a control when applying these assays to naturally-contaminated food samples (Chapter 7).

# CHAPTER 5

DEVELOPMENT OF A MOLECULAR ASSAY FOR THE DETECTION OF VIABLE *L. MONOCYTOGENES* IN FOOD SAMPLES

## 5.1 Experimental rational

Foodborne illness caused by most of the bacterial pathogens (*S. aureus* is a exception) requires the injestion of viable cells to cause illness. Therefore, when designing an assay to detect potential of contamination of food by bacterial pathogens it is essential to consider whether the analysis of the results can be equated with the potential of the food being unfit to eat. A food can be considered unfit if it contains viable cells of pathogenic bacteria such as *Campylobacter, Salmonella* or *E. coli* O157:H7.

The definition of what constitutes a viable cell remains contentious with the significance and precise physiological status of non-culturable organisms that retain some sign of viability still undetermined (Barer and Harwood, 1999). However, the viability status of a bacterial cell can be measured by assessing cellular integrity, the presence of specific nucleic acids or metabolic responsiveness (Keer and Birch, 2003). One proposed nucleic acid target for detecting viable bacteria cells is messenger RNA.

Messenger RNA (mRNA) molecules are an intermediary in protein synthesis and are only found in viable cells. It is also a highly labile with a very short half-life (minutes to seconds) and may provide a better indication of viability than DNA for the detection of bacterial pathogens (Bej *et al.,* 1991). Norton and Batt (1999) described the detection of viable *L. monocytogenes* in milk samples using a reverse transcription (RT) 5' nuclease PCR assay. In RT-PCR, a reverse transcriptase enzyme generates a complementary DNA copy (cDNA) of RNA molecules, which can then be amplified by the PCR. RT-PCR has also applied successfully to detect low numbers of *L. monocytogenes* in chicken meat enrichments (Navas *et al.,* 2005).

Koo and Jaykus (2000) developed a system where gene-specific reverse transcription was performed with the reverse oligonucleotides containing three mis-matched bases at the 3' end. This introduces a novel sequence in to the cDNA enabling it to be distinguished from the genomic DNA. The elongation

stage of the PCR is then performed at a sufficiently high temperature such that the oligonucleotides binds specifically only to the cDNA molecules thereby giving confidence that the amplification is a result of the mRNA and not genomic DNA. This approach was successfully applied to detect *L. monocytogenes* mRNA in reconstituted non-fat dry milk, with a detection limit of $10^4$ CFU in 25ml of sample (Koo and Jaykus, 2000).

An alternative method for detecting mRNA is nucleic acid sequence based amplification (NASBA) shown in Figure 5.1. NASBA uses three enzymes avian myoblastoma virus (AMV) reverse transcriptase, ribonuclease H, and T7 RNA polymerase to amplify targets. NASBA can be used to amplify from RNA in the presence of DNA (Compton, 1991), which may be advantageous for amplifying mRNA in the presence of genomic DNA. It has been applied to detect several food-associated pathogens including *C. jejuni* (Uyttendaele *et al.,* 1997), *L. monocytogenes* (Uyttendaele *et al.,* 1995) *S. enterica* (D'Souza and Jaykus, 2003) and *Bacillus* spp. (Gore *et al.,* 2003) .

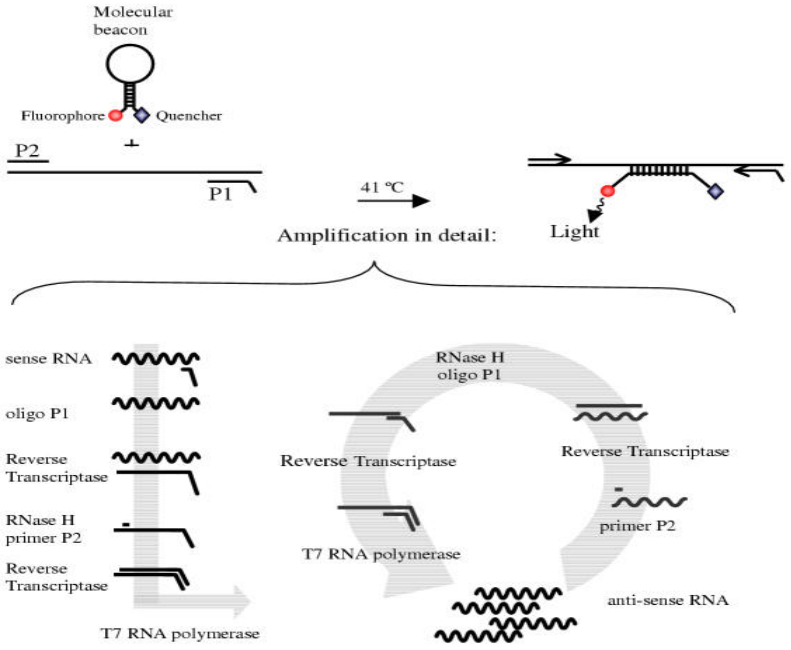



**Figure 5.1 NASBA amplification reaction with real-time molecular beacon detection of amplicons.**

Taken from Polstra *et al.* (2002) The overhang on the P1 encodes the promoter sequence for the T7 RNA polymerase. A molecular beacon with a fluorophore and a quencher was used in a NASBA as a real-time detection system.

In an alternative approach, Nogva *et al.* (2003) described a PCR and a DNA intercalating dye method that employed membrane integrity to prevent amplification of DNA from non-viable cells. The dye ethidium monoazide (EMA) selectively enters bacteria with damaged membranes where it becomes covalently linked to DNA by photo-activation. The irreversible binding of the dye to DNA prevents amplification and inhibits PCR from dead cells (i.e. those with impaired membrane potential). DNA from viable cells that exclude the dye is amplified giving a positive result in the PCR (Figure 5.2). The EMA PCR technique was compared to Baclight (viability staining; Molecular probes) and plate counts for *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* with a good correlation of viability to PCR signal (Nogva *et al.,* 2003). The technique has also been applied successfully for the detection of *C. jejuni* in poultry samples (Rudi *et al.,* 2005a) and *L. monocytogenes* in cheese (Rudi *et al.,* 2005b).

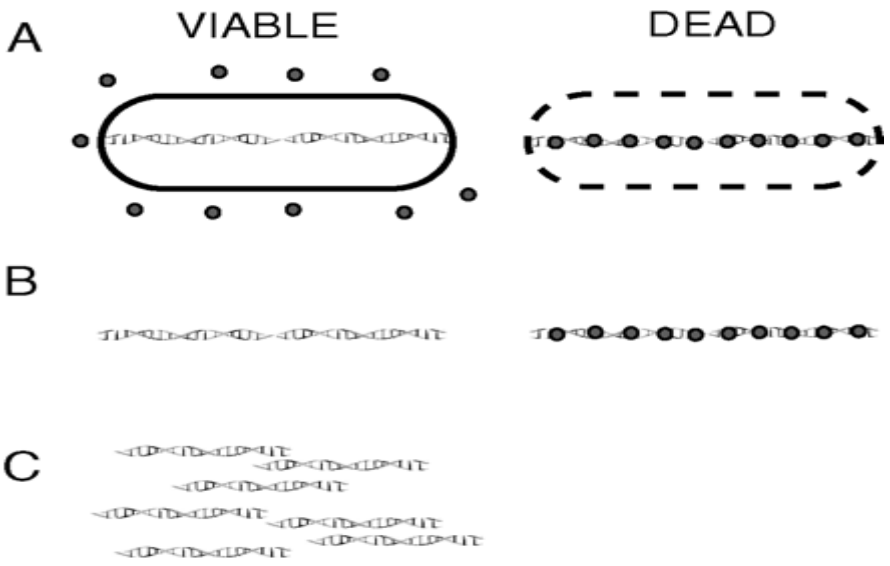



**Figure 5.2 EMA treatment applied to cells with an intact and non-intact cell membrane PCR as a means of detecting viable cells fromle cells.**

Taken from Rudi *et al.* (2005b) (A) EMA is added to the test sample containing both viable and dead cells. EMA penetrates the dead cells and binds to DNA. Light exposure for 1 min leads to covalent binding and inactivation of free EMA. EMA does not enter viable cells. (B) There are two populations of DNA after purification. The DNA population from viable cells is unstained, while the DNA from the dead cells is covalently bound to EMA. (C). the unstained DNA from viable cells is PCR amplified, while the DNA from dead cells with bound EMA cannot be amplified.

The definitive detection of viable cells in contaminated food represents a significant challenge in food microbiology. It was hypothesised that PCR and NASBA detection methods would be suitable for the detection of viable and non-viable cells (defined as growth of cells) in contaminated food samples. The specific objectives were to:

1) apply reverse transcriptase real-time PCR assays for the detection of viable *L. monocytogenes,*

2) investigate NASBA for the detection of viable *L. monocytogenes* and

3) Detection of viable *L. monocytogenes* cells using EMA treatment and 5' real-time PCR.

## 5.2 Results: Reverse transcriptase real-time PCR assays for the detection of viable *L. monocytogenes*

*L. monocytogenes* was selected as a model organism for the work given that a real-time PCR to detect the organism had been previously validated for use this study (Chapter 3). For this work culturable will be considered viable and non-culturable will be considered non-viable. Firstly, mRNA was investigated as a target molecule that when detected would indicate the presence of viable *L. monocytogenes* cells.

### 5.2.1 Comparison of one-step and two-step reverse transcription RT-PCR

To compare the one-step and two-step RT-PCR assays for this study, quantified control GADPH RNA supplied with the TaqMan gold kit was used to establish protocol parameters. RNA GAPDH target (50ng to 0.08ng) was amplified in the one-step (section 2.7.1. and 2.7.2) and two-step RT-PCR (section 2.7.1 and 2.7.3) to determine the sensitivity of the assays. A standard curve of mean $C_T$ value against concentration of RNA was plotted for both PCR reactions (Figure 5.3). The one-step reaction resulted in a lower $C_T$ of 19 compared to 22 for the two-step. The one-step reaction had a greater efficiency ($y = 2.3$) and had a higher linear correlation coefficient ($R^2 = 0.993$) than the two-step ($y = 1.7$ and $R^2 = 0.920$). Therefore, the one-step RT-PCR was chosen for subsequent RNA amplifications.

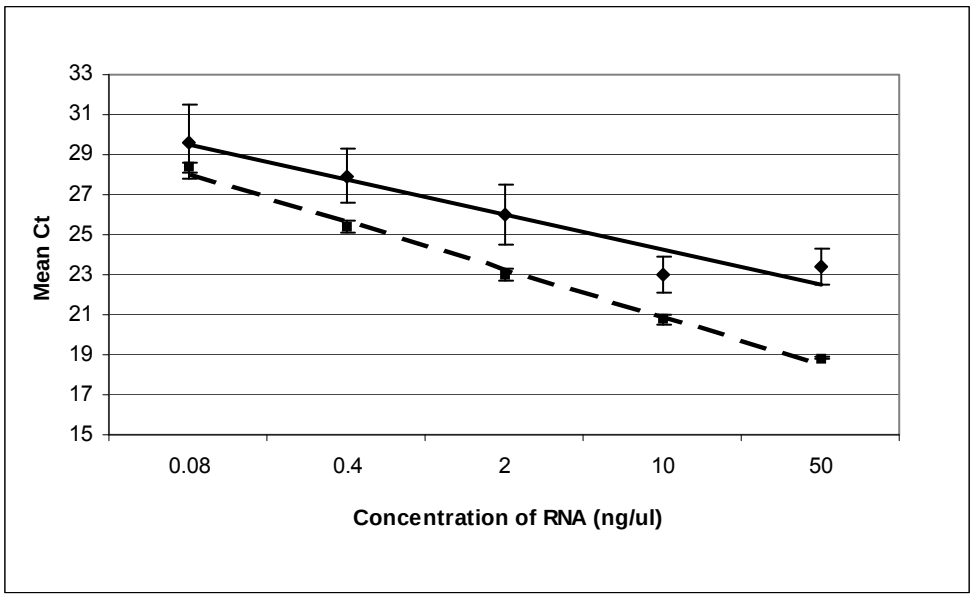



**Figure 5.3 Sensitivity of the one-step and two-step real-time RT-PCR applied to quantified GADPH RNA.**

A dilution series of quantified GADPH RNA was amplified in both RT-PCR assays. Standard curve plotted using concentration of RNA against mean cycle threshold ($C_T$). The straight lines calculated by linear regression and the one-step PCR (broken line) yielded a correlation coefficient ($R^2$) = 0.993 and slope of the graph (y) = 2.3. The two step PCR (solid line): yielded correlation coefficient $R^2$ = 0.920 and slope of the graph (y) = 1.7. Error bars indicate the standard deviations.

### 5.2.2 Detection of *L. monocytogenes* mRNA using RT-PCR

The *L. monocytogenes hlyA* PCR with a prior reverse transcriptase reaction to detect the mRNA was selected for the detection of *hlyA* mRNA. Validation, of the *L. monocytogenes hlyA* PCR has been described in Sections 3.3.1 to 3.3.3. *L. monocytogenes* NCTC 12724 cells in logarithmic growth phase (section 2.4.3) were prepared and an aliquot was autoclaved to provide a sample of non-viable cells as a negative control for the assay. The absence of viable cells in the autoclaved culture was confirmed by culture and no growth was observed after 48 h culture on Blood Agar. This material thereafter served as a control for all subsequent work. RNaqueous extraction (section 2.5.16) was applied to the *L. monocytogenes* cells in logarithmic growth phase.

The one-step RT-PCR method (section 2.7.1 and 2.7.2) was applied to nucleic acid extracted from both viable and non-viable *L. monocytogenes* cultures (Table 5.1). Using the RT PCR *L. monocytogenes hlyA* was detected from the viable culture and resulting in $C_T$ value of 26. However, *L. monocytogenes hlyA* was also detected from the non-viable culture with a slightly higher $C_T$ value of 32. The amplification of the non-viable cells was not expected, but could have been to be due to the presence of genomic DNA (gDNA) in the extracted nucleic acid giving a false positive result.

**Table 5.1 Detection of viable *L. monocytogenes* targeting mRNA using a RT-PCR.**

| Source of RNA | Detection of *L. monocytogenes* cells (mRNA) |
|---|---|
| Logarithmic cells (viable) | Detected (Ct 26) |
| Killed cells (non-viable) | Detected ($C_T$ 32) |
| Negative control | Not detected ($C_T$ 40) |

Negative control comprised of nuclease-free water; $C_T$ = cycle threshold; mRNA detected using RT-PCR and DNA using PCR.

To investigate if there was DNA in the extracted RNA samples they were applied to *L. monocytogenes hlyA* PCR without the RT step (section 2.9.7) (Table 5.2). *L. monocytogenes* cells DNA was detected in both the viable and non-viable cells with $C_T$ value of 26 and 30, respectively. This result indicated the was contaminating gDNA in the RNA preparations.

**Table 5.2 Application of PCR to RNA preparation from *L. monocytogenes* viable and non-viable cells.**

| Source of RNA | Detection of *L. monocytogenes* cells (DNA) |
|---|---|
| Logarithmic cells (viable) | Detected (Ct 26) |
| Killed cells (non-viable) | Detected ($C_T$ 30) |
| Negative control | Not detected ($C_T$ 40) |

Negative control comprised of nuclease-free water; $C_T$ = cycle threshold; mRNA detected using RT-PCR and DNA using PCR.

To reduce the presence of gDNA, RNA was extracted using an alternative method (RNeasy extraction, section 2.5.17) and that incorporated a DNase digestion, but amplification was still observed from DNA in the RNA extracts from viable cells (results not shown). This RT-PCR was abandoned it did not provide a definitive indication of the presence of viable cells.

### 5.2.3 **Development of an alternative RT-PCR protocol**

The approach of Koo and Jaykus (2000) using a reverse oligonucleotide containing three mis-matched bases at the 3' end in the RT step, which enables cDNA to be distinguished from the genomic DNA was investigated. The modification of the reverse oligonucleotide was applied to the existing *L. monocytogenes hlyA* RT-PCR.

The reverse oligonucleotide (called RThlyAR) was designed from a consensus sequence of the *L. monocytogenes hlyA* gene. The consensus sequence produced from published sequences (GeneBank accession numbers AY174657 to AY174669 and AL59197). These were aligned using ClustalW and the consensus derived from this. The oligonucleotide was designed to contain three consecutive mismatches near the 3' terminus so that it accounted for the variation observed in the genomic DNA sequences. The oligonucleotide sequence was subjected to a homology search using BLASTN software (section 2.11.4), which revealed no other identical sequence. The RThlyAR oligonucleotide in combination with hlyAF was used to amplify a 100 bp fragment of the *L. monocytogenes hlyA* gene encompassing the *hlyA* real-time PCR probe site.

To establish that the RThlyAR oligonucleotide did not amplify gDNA, MagNA Pure™ extracted *L. monocytogenes* NCTC 12927 DNA (section 2.5.1 and 2.5.2) was subjected to the *L. monocytogenes* PCR (section 2.9.7) using the newly designed RThlyAR oligonucleotide (position in gene and mismatches shown in Figure 8.2 of Appendix 1). A sample of *L. monocytogenes* DNA was also amplified using standard *L. monocytogenes* PCR with the hlyAR and hlyAR as a negative control (section 2.9.7).

Amplification (mean $C_T$ 20) was detected in the standard real-time PCR, but not in the sample amplified with the altered reverse oligonucleotide resulting in a mean $C_T$ 40. This result indicated that the oligonucleotide did not amplify genomic DNA and was suitable for the modified RT-PCR.

### 5.2.4 Detection of *L. monocytogenes* mRNA *hlyA* target using the modified RT-PCR

The altered oligonucleotide was used in the RT-PCR for the specific detection of *L. monocytogenes hlyA* mRNA targets. *L. monocytogenes* NCTC 12427 cells were grown to logarithmic phase in BHI Broth (section 2.4.3). An aliquot of broth culture was autoclaved for 15 min to kill the cells and generate a non-viable sample of *L. monocytogenes*. RNA was extracted from one ml of logarithmic phase culture and equivalent killed cells using RNeasy extraction with an extended (30 min) DNase digest (section 2.5.17). The resulting nucleic acid was amplified in the one-step RT-PCR and PCR (section 2.9.7). Amplification of target in the PCR resulted in a mean $C_T$ 31 and amplification of the target in the RT-PCR resulted in a mean $C_T$ 38 (Table 5.3). This was an improvement on the RT-PCR investigated in the previous experiment, however detection of mRNA in the non-viable cells indicated that this could be a false positive result. In addition, the modification of the assay has resulted in a loss of sensitivity, making the PCR unsuitable for use in food sample analysis where very low levels of detection are required.

**Table 5.3 RT-PCR and *hlyA* assay amplifications *L. monocytogenes* mRNA with altered RT oligonucleotide for logarithmic and killed cells.**

| Source of RNA | Detection of *L. monocytogenes* cells mRNA altered RT-PCR) |
|---|---|
| Logarithmic cells | Detected ($C_T$ 31) |
| Killed cells (non-viable) | Detected ($C_T$ 38) |
| Negative control | Not detected ($C_T$ 40) |

Negative control comprised of nuclease-free water; $C_T$ = cycle threshold.

## 5.3 Results: Investigation of Nucleic acid sequence based amplification (NASBA) or the detection of viable *L. monocytogenes*

RT-PCR used in the study was shown to be definitive for the detection of viable cells or sufficiently sensitive for application to food analysis. Therefore, Nucleic acid sequence based amplification (NASBA) an alternative approach to detection of mRNA in a background of DNA was investigated.

### 5.3.1 NASBA applied to *L. monocytogenes* mRNA *hlyA* target

A real-time NASBA assay oligonucleotides and a molecular beacon probe were designed to detect a target at the 3' end of the *L. monocytogenes hlyA* gene (positions in *hlyA* gene shown in Figure 8.1 of the Appendix) (section 2.7.4). Thermal denaturation profile studies were performed to determine the optimal annealing temperature of the molecular beacon probe. The thermal denaturation used the reagents for standard NASBA (section 2.7.6) with the addition of an oligonucleotide that was complementary to the beacon in place of the RNA sample. Reactions were prepared in triplicate containing either NASBA oligonucleotide or nuclease–free water as a non-template comparison. The samples were heated to 90 $^{o}$C for 5 min, and then the temperature was reduced at one degree per min in increments to 20 $^{o}$C. The Sequence Detection System recorded fluorescence at each temperature interval and calculated a thermal denaturation plot.

The change in fluorescence of a NASBA reaction containing the beacon and complementary single stranded oligonucleotide indicated that it was 100% homologous to the beacon. When no complementary target was added into the reaction, no fluorescence (above the background level) was detected below 50 $^{o}$C. In contrast when a target perfectly complementary to the beacon is available, the target and beacon hybridises and the hybrids fluoresce (Figure 5.4), as the temperature is raised the hybrid dissociates, accompanied by a marked decrease in fluorescence. At temperatures between 35 $^{o}$C and 50 $^{o}$C the probe-target elicited significantly stronger fluorescence than the probe alone

(Figure 5.5). These results implied that the probe was functioning and fluorescence was higher when the probe was bound to the target than when it was not at the temperature the reaction was performed (41 °C).

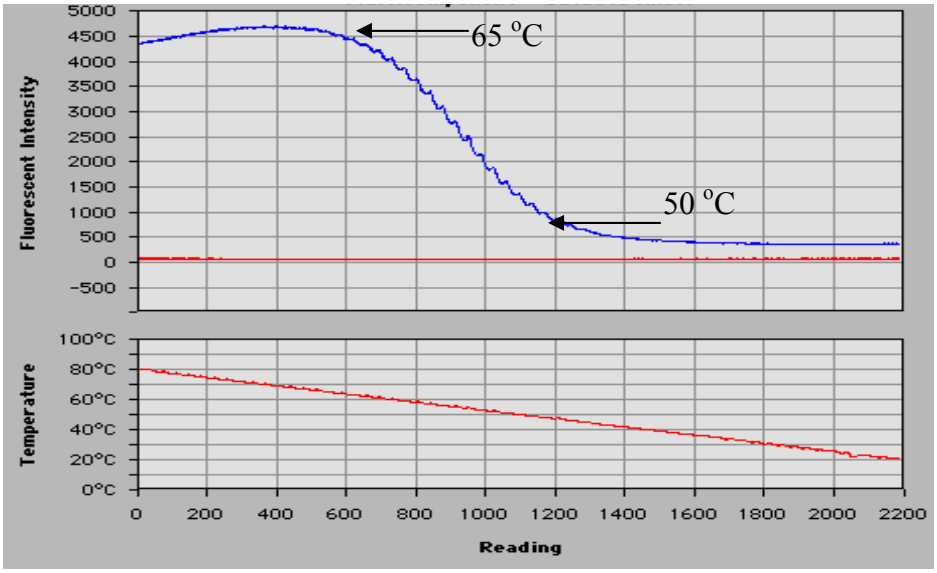



**Figure 5.4 Molecular beacon thermal denaturation in the absence of the complementary oligonucleotide.**

An increased fluorescent signal (blue line) was detected at temperatures above 50 °C generated from the beacon without complementary oligonucleotide binding. The red line in the upper plot represents the constant detection of the ROX internal reference dye. The lower plot shows the temperature of the reaction ranging from 80 °C to 20 °C.

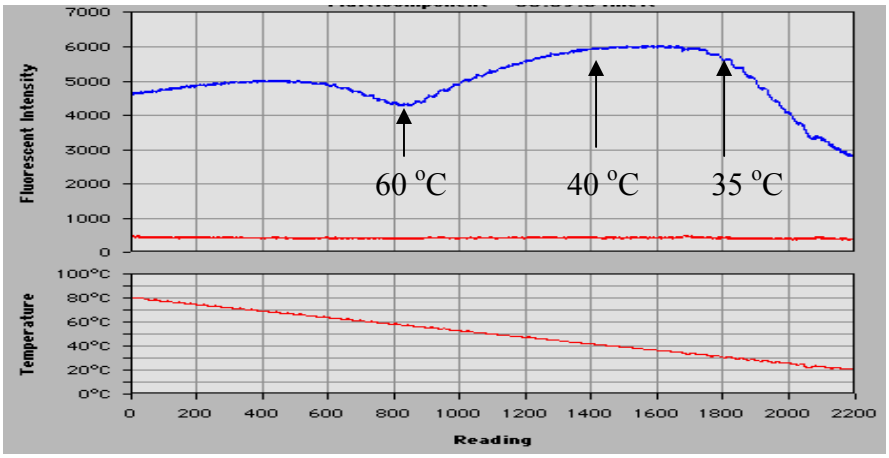



**Figure 5.5 Molecular beacon thermal denaturation in the presence of complementary oligonucleotide.**

The upper plot shows an increased fluorescent signal (blue) at temperatures between 35 °C to 40 °C, and constant detection of the ROX internal reference dye (red line). The lower plot shows the temperature of the reaction ranging from 80 °C to 20 °C.

### 5.3.2 **Real-time NASBA assay for the detection of *L. monocytogenes* mRNA *hlyA* target**

Nucleic acid was extracted from a culture of *L. monocytogenes* cells in logarithmic growth phase (section 2.4.3) by MagNA Pure™ Compact extraction (section 2.5.1 and 2.5.3) and subjected to real-time NASBA (section 2.7.6). No increase in fluorescence was observed during the reaction for any sample so it was not possible to determine if the reaction had worked.

### 5.3.3 **Standard NASBA assay for the detection of *L. monocytogenes* mRNA *hlyA* target**

To determine whether the product was being amplified but not detected by the real-time platform, the NASBA reaction was performed in real-time (section 2.7.6) and amplicons visualised using a gel-based detection system (section 2.9.4) but no product was detected. A possible cause of the lack of amplification may have been a problem with the oligonucleotides designed in this study. An alterative oligonucleotide set (BTP1 and BTP2) designed by Blais *et al.* (1997) for NASBA detection of *L. monocytogenes hlyA* gene was investigated, the oligonucleotide positions are shown in Figure 8.1 of the Appendix 1. The standard NASBA reaction (section 2.7.5) was applied to the nucleic acid extracted previously and products of the reaction visualised using a gel-based detection system (section 2.9.4). However, no amplicons were detected. This method was also unsuccessful in this work, suggesting it maybe a problem with sample preparation or the buffer system used during the reaction.

### 5.3.4 **Application of the NucliSENS Basic NASBA kit for detection of *L. monocytogenes hlyA* mRNA target**

A kit format of the reaction, as opposed to individually sourced and 'in-house' prepared reagents was investigated. A NucliSENS Basic kit for NASBA was modified (from electrochemiluminescence detection to probe detection as the instruments were not available at the time of this study) by the addition of a

molecular beacon for detection of the target mRNA. Nucleic acid was extracted from a culture of *L. monocytogenes* cells in logarithmic growth phase (section 2.4.3) by MagNA Pure™ Compact extraction (section 2.5.1 and 2.5.3) and applied to the NucliSENS kit (section 2.7.6). However, no amplification of the *L. monocytogenes hlyA* mRNA target was observed. The method was modified to amplify *L. monocytogenes* DNA, using an initial heating step to prime the DNA (section 2.4.11). An increase in fluorescence was detected indicating DNA could be amplified using the NucliSENS kit (Figure 5.6).

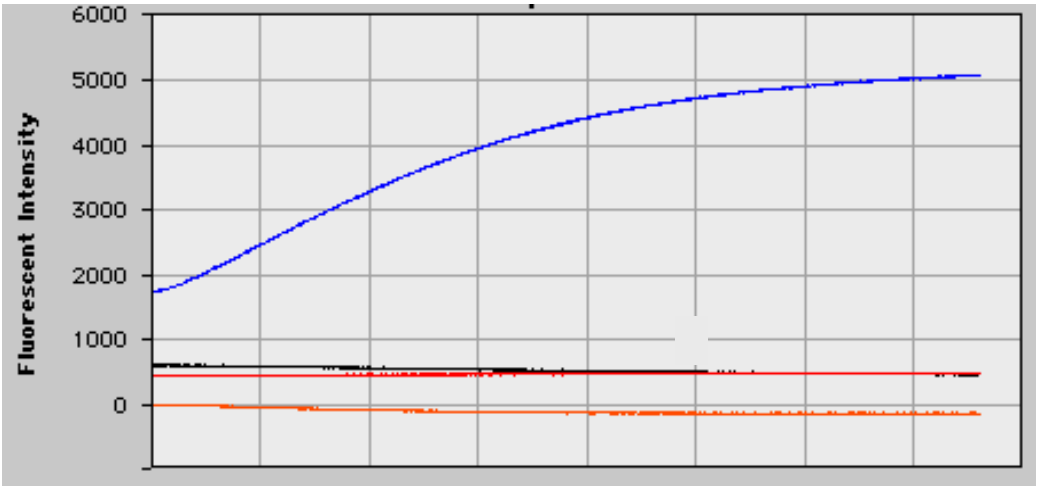



**Figure 5.6 Amplification of *L. monocytogenes* DNA in a real-time NASBA reaction**.

The blue line indicates an increase in fluorescence as amplification of the *L. monocytogenes* occurs in the real-time NASBA reaction. The red lines indicate the background fluorescence.

The findings from NASBA indicated that the reaction was successful for the DNA target but not mRNA. The RT-PCR used in this study was also unsuccessful. The failing could have been due to attempting to detect mRNA so this point, no further work was performed using RNA targets and a method for the specific amplification of DNA from viable cells was investigated.

## 5.4 Results: EMA PCR for the detection of viable *L. monocytogenes*

Nogva *et al.* (2003) described a novel method of preventing the amplification of DNA from non-viable cells in PCR. The method used a DNA intercalating dye ethidium monoazide (EMA), which can be covalently linked to DNA by photo-activation and this process prevent amplification. EMA combined with PCR was investigated was investigated as a method of detecting viable *L. monocytogenes*.

### 5.4.1 Optimisation of the EMA treatment and 5' real-time PCR using DNA.

EMA binds to DNA molecules not contained within an integral cell membrane and after photolysis, the EMA bound DNA can not amplified by the PCR due to intercalation. The optimal light source for photolysis and covalent attachment of EMA to DNA was first established. DNA was extracted from *L. monocytogenes* NCTC 12724 using MagNA Pure™ (section 2.5.1 and 2.5.2). Negative controls of nuclease-free water and a control of EMA without the addition of DNA and a positive DNA control except nuclease-free water was used in place of EMA solution were prepared. The DNA sample was incubated with EMA for 5 min and 10 min using each of four light sources: a 60 W table lamp and 11W, 22W and 500W fluorescent lamps (section 2.8.1). The DNA was washed and precipitated from solution using ethanol to remove unbound EMA, then amplified in the *L. monocytogenes* PCR (section 2.9.8).

Amplification was detected in DNA samples incubated with the 60 W table lamp, and 11W and 22W fluorescent lamps light sources, after incubation for 5 or 10 minutes, indicating that the EMA had not prevented amplification from the DNA. The 500W fluorescent lamp resulted in no amplification ($C_T$ undetected) indicating that EMA intercalation to the entire free DNA, preventing any amplification and hence this was used in subsequent experiments (Table 5.4). The PCR contained an internal positive control to monitor for any inhibition caused to the reaction by the EMA (IPC described

in Chapter 3). The IPC indicated that there was no inhibition ($C_T$ 26-30) of the PCR and the reaction had been successful in all samples treated with EMA.

**Table 5.4 EMA and DNA light source optimisation for detection of viable *L. monocytogenes* NCTC 12427 cells by PCR.**

| Light source | PCR result as mean $C_T$ (SD) | | | |
|---|---|---|---|---|
| | 5 min | | 10 min | |
| | *L. monocytogenes* | IPC | *L. monocytogenes* | IPC |
| 60 W table lamp | 31 (0.56) | 30 | 29 (0.18) | 31 |
| 11 W fluorescent | 27 (0.13) | 29 | 32 (0.46) | 30 |
| 22 W fluorescent | 29 (0.70) | 30 | 29 (0.45) | 30 |
| 500W fluorescent | >40 (0) | 30 | >40 (0) | 30 |
| Negative | >40 (0) | 30 | >40 (0) | 30 |
| Positive control | 22 (0.13) | 30 | 22 (0.13) | 30 |

DNA samples treated with ethidium monoazide exposed to different light sources amplified in the *L. monocytogenes hlyA* IPC assay. $C_T$ = cycle threshold.

To establish the optimal photolysis time of EMA with DNA, the previous experiment was repeated using only the 500W fluorescent light and incubation times of between 15 s to 5 min (Table 5.5). Amplification was detected in samples that had been incubated for 15s, 30s and 45s and resulted in $C_T$ values of 34, 35 and 37 respectively. No amplification was detected in samples where the DNA had been incubated for 60s or longer. Incubation of the sample with EMA for 60 s was sufficient to prevent amplification of free DNA in the PCR assay. However, to ensure the EMA was irreversibly bound and unbound EMA was photolysed, an incubation of 120 s was chosen for subsequent reactions.

**Table 5.5 EMA and DNA incubation time optimisation for detection of viable *L. monocytogenes* NCTC 12427 cells by PCR.**

| Incubation time (s) | PCR result as mean $C_T$ (SD) | |
|---|---|---|
| | *L. monocytogenes* | IPC |
| 15 | 34 (1.02) | 30 |
| 30 | 35 (0) | 30 |
| 45 | 37 (2.32) | 30 |
| 60 | >40 (0) | 30 |
| 90 | >40 (0) | 30 |
| 120 | >40 (0) | 30 |
| 180 | >40 (0) | 30 |
| 240 | >40 (0) | 30 |
| 300 | >40 (0) | 30 |
| Positive control | 29 (0.44) | 30 |
| Negative EMA control | >40 (0) | 30 |
| Negative control | >40 (0) | 30 |

DNA samples treated with ethidium monoazide exposed a 500W fluorescent light amplified in the *L. monocytogenes hlyA* IPC assay. $C_T$ = cycle threshold.

### 5.4.2 Detection of viable *L. monocytogenes* cells using EMA treatment and 5' real-time PCR.

The optimised EMA PCR was applied to the specific amplification of DNA from viable *L. monocytogenes* cells. *L. monocytogenes* NCTC 12427 cells were grown overnight in BHI Broth and designated as 'viable cells and an aliquot of cells were autoclaved for 15 min and designated as 'killed cells' (non-viable) (section 2.4.3). The ACC of viable cells was determined using BN Agar plates and incubation for 48 h at 30 $^{o}$C (section 2.4.2). Non-viability of killed cells was confirmed using BN plates and incubation for 24 and 48 h at 30 $^{o}$C after which no cell growth was observed. A one ml aliquot of viable cells and killed cells were subjected to EMA treatment (section 2.8.2) and DNA extraction (section 2.5.10). The resulting DNA was amplified in the *L. monocytogenes* PCR (section 2.9.8). A positive DNA control was prepared in an identical way except nuclease-free water was used in place of EMA solution. Negative controls of nuclease-free water and a control of EMA without the addition of bacterial cells were also prepared. Triplicates of each sample were subjected to EMA and duplicates of each were amplified in the PCR assay (Table 5.6).

**Table 5.6 Amplification of *L. monocytogenes* NCTC 12427 DNA from viable and killed cell samples treated with and without EMA.**

| Sample type | PCR result (mean $C_T$) | |
|---|---|---|
| | Plus EMA | Minus EMA |
| Viable cells | 18 | 16 |
| Killed cells | >40 | 19 |
| Negative control | >40 | >40 |
| Positive control | 15 | 15 |

$C_T$ = cycle threshold; EMA: ethidium monoazide.

No amplification ($C_T$ >40) was observed in the DNA sample from killed cells treated with EMA. Amplification was detected in DNA samples from viable cells treated either with EMA ($C_T$ 18) or without EMA ($C_T$ 16) and killed cells without EMA treatment ($C_T$ 19). A two $C_T$ loss in sensitivity was observed after cells were treated with EMA, however, these results indicated that the assay was successful for the detection of viable cells.

## 5.5 Discussion

The aim of the work described in this chapter was to establish a sensitive, robust and rapid molecular method for the detection of viable bacterial pathogens in food samples. Given the rapidity of molecular-based methods such as RT-PCR, NASBA and EMA PCR, these were investigated for use with complex whole food samples. Initially, mRNA was investigated as a target for the detection of viable *L. monocytogenes* in food samples. A real-time RT-PCR was chosen to amplify mRNA targets because of the increased sensitivity and specificity achieved with fluorescent probe detection of amplicons compared to block-based PCR. Single-step and two-step protocols were compared to establish which was the most sensitive for amplifying RNA target. In the one-step format, the RT reaction and PCR amplification take place sequentially in a single tube. The two-step protocol reverse transcribes the RNA in one reaction and then a small proportion of this reaction (in this case ten percent) is subjected to PCR amplification in a second tube. One-step

assays must be sensitive and specific because each reaction cannot be separately optimised, unlike the two-step assay.

### 5.5.1 **Reverse transcriptase real-time PCR assays for the detection of viable *L. monocytogenes***

One and two-step real-time RT-PCR assays were compared and the one-step was found to be more sensitive. This is in keeping with a recent study by Tsai *et al.* (2006) who also reported a one step real-time RT-PCR to be more sensitive when applied to the detection of viable *E. coli* O157:H7 in food samples. The detection of viable *L. monocytogenes* in bacterial culture samples using a reverse transcription 5' nuclease PCR assay has been previously described by Batt *et al.* (1997). RT-PCR has also been used to detect low numbers of *L. monocytogenes* in chicken meat enrichments (Navas *et al.,* 2005). The RT-PCR for the detection of viable *L. monocytogenes* bacteria was unsuccessful in our study, due to the detection of false positive PCR amplification generated from non-viable cells. Contaminating genomic DNA from the nucleic acid extraction may have caused the false positive. In order to investigate the possibility, an extended DNase digest was applied to the extracted nucleic acid, however amplification was still detected from DNA.

Navas *et al.* (2005) investigated the use of DNase I enzyme concentrations for removing DNA from *L. monocytogenes* lysates and reported that only when *L. monocytogenes* lysates contained $\leq 10^5$ $CFUml^{-1}$ did the DNase treatment prove successful. Navas *et al.* (2005) also applied RT-PCR and PCR to poultry samples artificially spiked with *L. monocytogenes,* only the samples inoculated with $10^2$ CFU per PCR or greater were detected by the PCR and they concluded that 24-hour enrichment was necessary to detect samples containing fewer cells. However, by incorporating an enrichment stage to resuscitate and amplify the viable cells to a detectable level negates the need to detect mRNA, as only viable cells will be increased by the culture stage and so detected in the

assay. However, when using a cultural enrichment stage, the assays are no longer rapid, as they will take days to return results.

The altered oligonucleotide RT-PCR as described by Koo and Jaykus (2000) was also investigated for the specific detection of *L. monocytogenes* mRNA targets. The altered oligonucleotide resulted in an improved mRNA detection, however the assay did not provide conclusive evidence that the signal detected is reliably generated by the amplification resulting from reverse transcribed RNA and not genomic DNA. From this work, it was concluded that this method was also unsuitable for the detection of mRNA as a marker of viability. This was probably due to low levels of gDNA still being present in the extracted nucleic acid preparation being amplified by the very sensitive PCR despite the DNase digest (lowest level of detection equal to 15 CFU per PCR).

### 5.5.2 Investigation of NASBA for the detection of viable *L. monocytogenes*

When using viability assays that use molecular detection techniques, particular attention needs to be applied to the correlation between molecular findings and culture-based viability. Gene expression levels can vary considerably and their levels may be affected by the choice of incubation medium, the matrix extracted from and the treatment of cells during extraction. In addition, the area of the gene targeted by the PCR may not be indicative of viability. An alternative method, NASBA, was then investigated as an assay that could be applied to specifically amplify RNA in a background of DNA and hence avoid the false positive results found using RT-PCR.

Traditionally, NASBA used ECL for the detection of amplified products, but it has also been adapted for real-time amplification (Leone *et al.,* 1998). In this study, both a conventional and real-time NASBA were investigated but no amplification products were detected by either method. The RNase H was changed from the cloned version to *E. coli* RNase H, and the supplier of

AMV-RT was changed from Roche to Seikagaku as NASBA reactions can be sensitive to the brand of enzymes used (Sooknanan *et al.,* 1995); however, the NASBA reaction remained unsuccessful. The reasons for this were unclear, although Nadal *et al.* (2007) reported that RNA molecules tend to adopt secondary structures, this can be prevented by heating the template and causing the bonds forming the secondary to disassociated. However, the NASBA reaction used a temperature of 41 $^{o}$C that was too low for preventing secondary structures. If so, the formation of secondary structures in the *hlyA* RNA transcript would most likely have prevented the oligonucleotides or probe binding and therefore prevented amplification. The level of *L. monocytogenes* gene expression can be affected by incubation media (Bron *et al.,* 2006) and food matrix (Hanna and Wang, 2006) and thus the mRNA target might not be a suitable target for a diagnostic PCR for detecting *L. monocytogenes* in the wide range of food types regularly encountered in food microbiology testing laboratories.

### 5.5.3 **Application of EMA PCR for the detection of viable *L. monocytogenes***

Difficulties were encountered when detecting *L. monocytogenes* mRNA in our study and so a DNA marker was investigated as an alternative target. DNA is a stable molecule that can remain detectable after cell death so is not generally used as a marker of viability. However, by detecting only the DNA present in bacterial cells it may be possible to detect viable cells, which were defined for the purpose of the EMA work, as bacterial cells with an intact membrane. EMA dye was used to prevent PCR amplification from 'free' DNA (i.e. not contained within a bacterial cell). In this study, preliminary results from the EMA–PCR method indicated the PCR signal was only generated from viable cells therefore making this assay suitable for detecting viable bacteria. This correlates with the results obtained by Rudi *et al.* (2005b; 2005a), who applied EMA–PCR for the detection of viable and dead *L. monocytogenes* in Gouda-like cheeses. The authors suggested that this technique could solve the problem of discriminating between viable but not culturable state and dead bacteria.

Rueckert *et al.* (2005) compared EMA and DNase I treatment for the selective detection and enumeration of the total and viable vegetative cell and spore content of *Anoxybacillus flavithermus* in milk powder by PCR. The author reported that EMA caused considerable cell inactivation, whereas DNase I treatment had no effect on cell viability so was the preferred technique. However, this experiment was performed with dehydrated samples and this may have affected the ability of viable cells to efflux the EMA. EMA was shown to penetrate viable cells and cause a loss of genomic DNA (Nocker and Camper, 2006), which could lead to an underestimation or failure to detect viable cells in a sample. This may be dependent on type of organism the assay is applied to, its physiological state and the sample matrix investigated.

## 5.6 Conclusion

The hypothesis that was tested in this work was: can PCR and NASBA detection methods can be used to indicate definitively the presence of viable bacterial cells in a contaminated food sample? In this study, mRNA targets were not successfully detected and so could not be used to indicate definitively, the presence of viable *L. monocytogenes* cells when used as a target molecule in RT-PCR or NASBA reactions. However, EMA treatment combined with real-time PCR was shown to be a rapid and simple method of detecting viable *L. monocytogenes* that would be applicable to detection of the organism in contaminated food samples. The EMA method is also advantageous, as is generic and could be applied to other foodborne pathogenic bacteria. Future work should include assessment of EMA to other foodborne bacteria and application of the method to naturally-contaminated food samples.

# CHAPTER 6

## DEVELOPMENT OF PCR ASSAYS FOR THE DETECTION OF BACTERIAL PATHOGENS IN FOODS.

## 6.1 Experimental rational

*Campylobacter* spp*., Salmonella* spp., *Clostridium perfringens, Escherichia coli* O157:H7 and *Listeria monocytogenes* are the bacterial pathogens that cause the greatest disease burden in the England and Wales (Adak *et al.,* 2002). Because these bacterial pathogens cause significant disease burden and are associated with food contamination, they represent suitable target organisms for a PCR for food hygiene screening of food samples. In addition *Staphylococcus aureus* is an important pathogen due to its ability to produce heat-stable enterotoxins that cause gastrointestinal illness and it is also an indicator of bad hygiene practices (Peles *et al.,* 2007).

The study aimed to use PCR assays to detected *Campylobacter* spp*., Salmonella* spp. *C. perfringens, E. coli* O157:H7, *L. monocytogenes, S. aureus* and an internal positive control target. Two PCR assays were previously described in this study: PCR for the detection of *L. monocytogenes* was described in chapter 3 and a PCR to detect the green fluorescent protein (*gfp)* gene PCR used as the internal positive control (chapter 4). Two previously published duplex real-time PCR assays, were selected: a speciation assay for the detection of *C. jejuni* and *C. coli* previously described by Best *et al.* (2003) and a novel assay for the detection of *C. perfringens* (Amar *et al.,* 2005) have been applied in clinical laboratories at the Health Protection Agency. All these PCRs used identical PCR reagent and cycling conditions and so it could be possible to multiplex these reactions in one assay. However, PCR assays for the detection of *E. coli* O157:H7, *Salmonella* spp. and *Staphylococcus aureus* were not available at this time and suitable assays and target genes were required.

One of the virulence determinants of *E. coli* O157:H7 is the intimin gene (*eae*) found in the locus of enterocyte effacement, which is essential for intimate attachment to epithelial cells by formation of the attaching–effacing lesion (Nataro and Kaper, 1998). At least four different intimin types have been described and designated α, β, δ, and γ (Adu-Bobie *et al.,* 1998). The gamma

intimin gene (*eaeγ*) encodes a 94-97 kDa outer membrane protein (intimin) responsible for adherence on the intestinal surface (Beebakhee *et al.,* 1992). The *eaeγ* is situated at the distal end of the *eae* gene and can vary greatly among the EHEC and so PCR assays targeting this gene have been developed to specifically detect EHEC O157:H7 isolates (Gannon *et al.,* 1993; Louie *et al.,* 1994; Willshaw *et al.,* 1994).

At present, the most common target for real-time PCR detection of *Salmonella* spp. is the invasion A (*invA*) gene that is present in all *Salmonella* species. *Salmonella* are divided into two species: *S. enterica* which includes six subgroups; and *S. bongori* (Herrera-Leon *et al.,* 2005). The *iroB* gene was first isolated from *S. enterica* serovar Typhi and is present in all known *S. enterica* serovars (Mills *et al.,* 1995), but is absent from *S. bongori* and so can be used for the identification of *S. enterica* (Baumler *et al.,* 1997). *IroB* is a glycosyltransferase that glycosylates enterochelin to produce salmochelin. This mechanism is a defence strategy of virulent strains to restore an iron acquisition system in response to enterochelin appropriation by mammalian siderocalin (Mercado *et al.,* 2008).

Potentially pathogenic *Staphylococcus aureus* strains in food samples are identified by their ability to coagulate plasma and to produce a thermostable nuclease (Chesneau *et al.,* 1993). To specifically identify *S. aureus* species using genotypic analyses, the thermostable nuclease (*nuc*) gene has been used as a target for identification (Brakstad *et al.,* 1992; Chesneau *et al.,* 1993; Pinto *et al.,* 2005; Wilson *et al.,* 1991).

Multiplex PCR can be used to analyse a food sample for several different bacterial pathogens or indicator organisms simultaneously. The multiplex PCR uses oligonucleotide sets within a single reaction tube to amplify several nucleic acid fragments from gene targets (Abubakar *et al.,* 2007). For example, the *E. coli* (β-glucuronidase gene), *S. aureus* (thermonuclease gene) and *Salmonella* spp. (replication origin sequence target) were detected in a single-tube multiplex real-time PCR assay (Elizaquivel and Aznar, 2008). An

important consideration for the design of multiplex PCR for the detection of foodborne pathogens is the sensitivity of the assay. Multiplexing oligonucleotide sets can result in loss of sensitivity due to competitive inhibition between the oligonucleotides (You *et al.,* 2008). PCR sensitivity and specificity can be improved by including an additional phase of PCR either nested or hemi-nested PCR. Hemi-nested PCR has been used in multiplex to amplify a large number of targets (>400) with increased sensitivity as compared to un-nested PCR (Dear *et al.,* 1998). A nested PCR was used to detect *L. monocytogenes* in milk which resulted in a detection limit of 5 -10 CFU in 25ml, that is extremely sensitive (Herman *et al.,* 1995).

Multiplex assays do not usually exceed six oligonucleotide sets because of the limitations in the ability to resolve many fragments in agarose and the potential for generating non-specific products that make interpretation difficult (Call *et al.,* 2003). DNA or oligonucleotide microarrays have been used for detecting amplicons from multiplex PCR. For example, a Luminex suspension bead array was used to simultaneously detect *E. coli, Salmonella* spp*., L. monocytogenes* and *C. jejuni* gene fragments amplified using a multiplex PCR targeting the 23S rRNA gene (Dunbar *et al.,* 2003).

The Luminex suspension bead array using Bio-plex software will be investigated for detection of PCR amplicons. This suspension bead array uses polystyrene microspheres containing internal dyes with two spectrally distinct fluorochromes, which allow each microsphere set to be distinguished by its spectral address (Dunbar *et al.,* 2003). Each microsphere set can possess a different reactant on its surface for example the microspheres can be hybridized to PCR amplicon (Figure 6.1). By adding a streptavidin-phycoerythrin reporter, all hybridized amplicons captured by their complementary nucleotide sequence on the microbeads are recognized and the median fluorescence intensity (MFI) is subsequently measured by flow cytometry (Landlinger *et al.,* 2009).

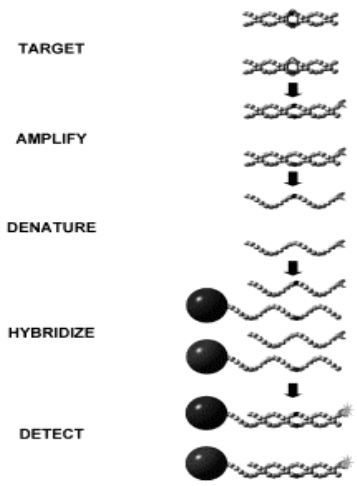



**Figure 6.1 Diagram of PCR amplicon and Bio-plex probe hybridization assay**

Adapted from Dunbar *et al.* (2003). The DNA target is amplified and a phycoerythrin reporter is added to the amplicon. The strands are denatured and a probe containing a complementary sequence to the target and a polystyrene microsphere hybridises to the DNA target. The target bound to the probe is detected by excitation of fluorochromes within the microsphere in the presence of phycoerythrin.

Culture detection of foodborne bacterial pathogens is labour-intensive, slow and currently not able to meet the demands of rapid detection of bacterial pathogens in contamination of food samples. PCR assays can offer rapid, specific and sensitive alternative method of detection of a range of foodborne pathogens simultaneously. To test this hypothesis PCR assays were designed or modified to enhance their robustness and reliability and then applied to the detection of bacterial pathogens directly in food samples. The hypothesis was investigated by developing a novel multiplex PCR assay for simultaneous detection of *L. monocytogenes, E. coli* O157:H7, *S. enterica, C. jejuni, C. coli, C. perfringens, E. coli* GFP and *S. aureus*. This was then examined for its rapidity, sensitivity and specificity.

The specific objectives were to:

1) design real-time PCR assays for the detection of *E. coli* O157:H7, *S. enterica* and *S. aureus* and validate existing real-time PCR assays for use in this work,

2) develop hemi-nested and fully nested PCR to increase the sensitivity of the PCR assays and

3) multiplex the hemi-nested PCR assays with Bio-plex suspension array detection of the amplicons.

## 6.2 Results: Development and validation of Real-time PCR assays

In this chapter, the development of novel PCR assays for the detection of *E. coli* O157:H7, *S. enterica* and *S. aureus* are described. The novel PCRs and published assays for detecting *C. perfringens*, *C. jejuni* and *C. coli,* were validated for use in this study. Unless stated DNA was extracted using MagNa Pure (section 2.5.1 and 2.5.2) for all experiments in this chapter. The PCR conditions for all reactions in section 6.2.1 to 6.2.7 are given in section 2.9.7. All validation i.e. sensitivity and lowest level of detection; nested and hemi-nested development; and Bio-plex development was performed using NCTC cultures described in section 2.1.1. For inclusivity and exclusivity, and application of the nested assay experiments the cultures or samples used are described in detail, in the relevant results sections.

### 6.2.1 Development of the eaeγ PCR for detection of *E. coli* O157:H7

A PCR for the detection of *E. coli* O157:H7 was designed that targeted the intimin gene (*eaeγ*). The PCR was challenged with a ten-fold serial dilution of quantified (section 2.9.14) DNA extracted from *E. coli* NCTC 12900 to determine its sensitivity. The PCR was 100% efficient (y = -3.34) and linear ($R^2$ = 0.994) over a seven-log dilution range and had a limit of detection of 17 genome copies per PCR (Figure 6.2). To evaluate the specificity of the intimin *γ* PCR assay, it was challenged with 51 DNA extracts. The *Escherichia*, *Shigella*, *Yersinia* & *Vibrio* Reference Unit (HPA, Colindale, London) provided 50 DNA extracts derived from patient samples. The reference lab had culture and PCR confirmed all the isolates as enteropathogenic *E. coli.* The intimin *γ* (*eaeγ*) gene fragments had been detected in 40 samples and were confirmed as serotype O157:H7. Ten *E. coli* were enteropathogenic but not reactive with the *eaeγ* gene PCR or probe. The assays used were described by Willshaw *et al.* (1994).

Using the PCR designed in this study (section 2.9.7) the *eaeγ* gene fragment was amplified from all the DNA extracts with a mean $C_T$ 20 (SD 3.33). There

was no amplification ($C_T$ 40) from DNA extracts of *eaeγ* negative samples. These results indicated that the PCR was specific for the intimin γ (*eaeγ*) gene target, which in itself is specific to *E. coli* O157:H7.

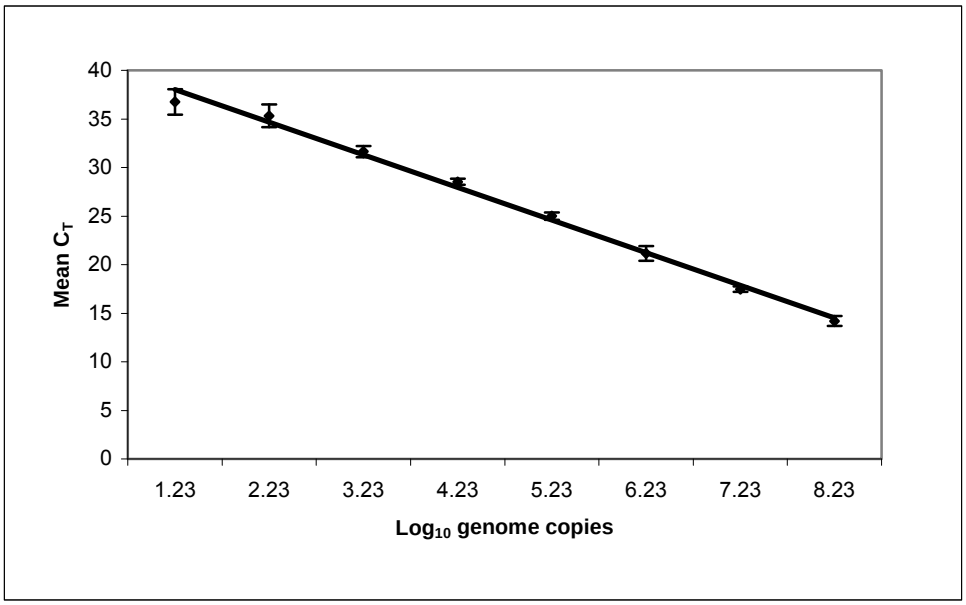



**Figure 6.2 Sensitivity of the eaeγ real-time PCR applied to DNA isolated from *E. coli* O157:H7 NCTC 12900.**

A ten-fold serial dilution of quantified DNA was amplified using *eaeγ* specific oligonucleotide and probe sets. Standard curve was plotted using $Log_{10}$ genome copies per PCR against mean $C_T$. The straight lines calculated by linear regression and the data (solid line) yielded a correlation coefficient ($R^2$) = 0.994 and slope of the graph (y) = -3.4. Error bars indicate the standard deviations based on three replicate PCR results.

### 6.2.2 Development of the iroB PCR for the detection of *S. enterica*

A PCR for the detection of *S. enterica* was designed that targeted the *iroB* gene. The PCR was challenged with a ten-fold serial dilution of quantified DNA (section 2.9.14) extracted from *S. enterica* NCTC 13349 to determine its sensitivity (Figure 6.3). The *iroB* PCR lacked efficiency at 83% (y = -4.5), but was a sensitive PCR with a limit of detection of seventy genome copies per PCR. To increase the PCR efficiency, the assay was modified by lowering the probe concentration from 100 nM per reaction to 30 nM which resulted in an increased efficiency of 93% (y = -3.7), an acceptable $R^2$ = 0.990 and maintained the sensitivity.

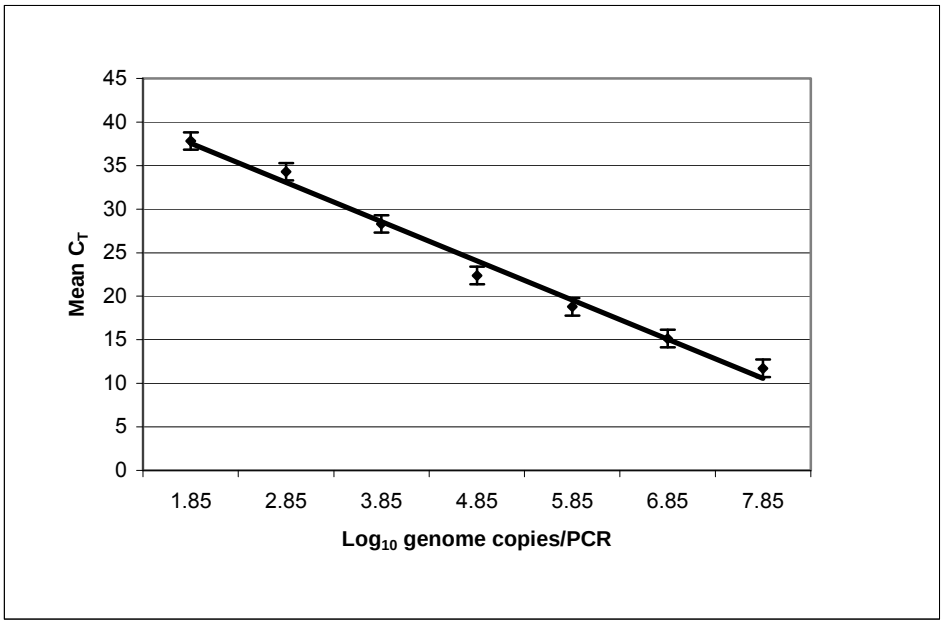



**Figure 6.3 Sensitivity of the iroB real-time PCR applied to DNA isolated from *S. enterica* DNA NCTC 13349.**

A ten-fold serial dilution of quantified DNA was amplified using *iroB* gene specific oligonucleotide and 20nM probe sets. Standard curve was plotted using $Log_{10}$ genome copies per PCR against mean cycle threshold ($C_T$). The straight lines calculated by linear regression and the data (solid line) yielded a correlation coefficient ($R^2$) = 0.990 and slope of the graph (y) = -3.7. Error bars indicate the standard deviations based on three replicate PCR results.

Specificity of the iroB PCR for the detection of *S. enterica* was then determined. The iroB PCR was challenged with six *S. enterica* NCTC cultures and 86 wild-type isolates. The wild-type isolates were provided by the Salmonella Reference Laboratory (HPA, Colindale, London) derived from patient samples and food isolates. The reference lab had culture confirmed all the isolates as *Salmonella* spp.

All the samples were tested in duplicate, all of which were successfully amplified with a mean $C_T$ 13 (SD 3.0). There was no amplification detected ($C_T$ 40) from a culture of *S. bongori* (NCTC 12419), which does not contain the *iroB* gene.

### 6.2.3 **Development of the nuc PCR for the detection of *S. aureus***

A real-time PCR for the detection of *S. aureus* was designed that targeted the *nuc* gene. The PCR was challenged with a ten-fold serial dilution of quantified DNA (section 2.9.14) extracted from *S. aureus* NCTC 6571. The sensitivity of the nuc PCR was calculated as 10 genome copies per PCR. The nuc PCR was 100% efficient (y = -3.3) and linear ($R^2$ = 0.991) over a seven log dilution range (Figure 6.4). DNA samples were tested in four replicates and concordant results were obtained. Specificity of the nuc PCR for the detection of *S. aureus* was determined using 105 wild-type *S. aureus* isolates provided and culture confirmed by The Food Safety Microbiology Laboratory (FSML), HPA and two NCTC cultures (6571 and 1803), so in total 107 samples were tested. This resulted in amplification of the *nuc* gene from all DNA samples with a mean $C_T$ of 18 (SD 2.64).

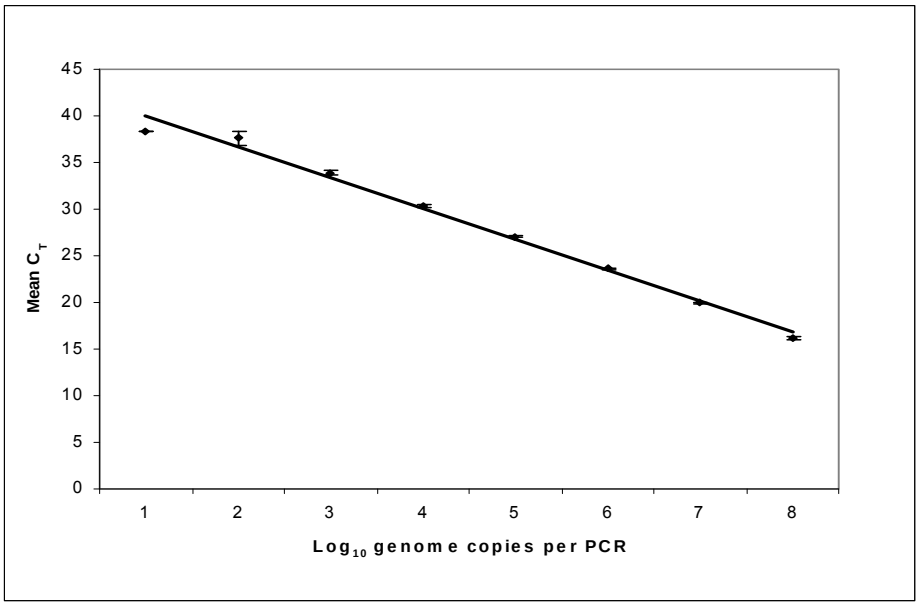



**Figure 6.4 Sensitivity of nuc real-time PCR assay applied to DNA isolated from *S. aureus* NCTC 6571.**

A ten-fold serial dilution of quantified DNA was amplified using *nuc* gene specific oligonucleotide and probe sets. Standard curve was plotted using $Log_{10}$ genome copies per PCR against mean cycle threshold ($C_T$). The straight lines calculated by linear regression and the data (solid line) yielded a correlation coefficient ($R^2$) = 0.991 and slope of the graph (y) = -3.3. Error bars indicate the standard deviations based on four replicate PCR results.

### 6.2.4 **Validation of the ceuE PCR for detection of *C. coli***

A real-time PCR For the detection of *C. coli* that targeted a 103 bp fragment of the Periplasmic binding protein (ceuE) was selected. PCR sensitivity was measured using DNA extracted from *C. coli* NCTC 12110. The lowest level of detection for the ceuE PCR was one genome copy per PCR. The PCR was linear over seven logs with y = -3.562, a $R^2$ = 0.996 resulting in an efficiency of 95%. The assay was tested with 17 *C. coli* confirmed isolates that had been supplied by The Campylobacter and Helicobacter Reference Unit (CHRU), HPA and amplification was detected from DNA extracted from all isolates with a mean $C_T$ of 14 (SD 2.61).

### 6.2.5 **Validation of the mapA PCR for detection of *C. jejuni***

A real-time PCR for the detection of *C. jejuni* that targeted a 96 bp fragment of the Membrane associated protein A (*mapA*), was selected. PCR sensitivity was measured using DNA extracted from *C. jejuni* NCTC 11168. The lowest level of detection for the mapA PCR was 3375 genome copies per PCR. The PCR was linear over seven logs with y = -3.607, $R^2$ = 0.989 resulting in an efficiency of 95%. The assay was tested with 23 *C. jejuni* confirmed isolates that had been supplied by The Campylobacter and Helicobacter Reference Unit (CHRU), HPA and amplification was detected from DNA extracted from all isolates with a mean $C_T$ of 17 (SD 4.44).

### 6.2.6 **Validation of the PCR for the detection of *C. perfringens* alpha-toxin gene and entertoxin gene**

The PCR assays for the detection of *C. perfringens,* was published as a duplex assay targeting two gene fragment: *C. perfringens* the *cpa* and *cpe* genes. However, each gene target was validated as monoplex assay to establish the levels of sensitivity and specificity in this study. The *C. perfringens* alpha-toxin gene (*cpa*) reaction amplified a 103 bp fragment of the gene. PCR sensitivity was measured using DNA extracted from *C. perfringens* NCTC 8239. The lowest level of detection in the *cpa* PCR was 341 genome copies

per PCR. The PCR was linear over five logs y = -3.518, $R^2$ = 0.992 resulting in an efficiency of 96 %. The assay was tested with 39 *C. perfringens* confirmed isolates that had been supplied by FSML, HPA and amplification was detected from DNA extracted from all isolates with a mean $C_T$ of 17 (SD 1.7).

The *C. perfringens* enterotoxin gene (*cpe*) reaction amplified a 103 bp fragment of the gene. PCR sensitivity was measured using DNA extracted from *C. perfringens* NCTC 8239. The lowest level of detection in the cpe PCR was 341 genome copies per PCR. The PCR was linear over five logs with y = -3.504, $R^2$ = 0.997 resulting in an efficiency of 96 %. The assay was tested with 33 *C. perfringens* isolates that contained *cpe* and *cpa* gene fragments, which had been supplied by FSML, HPA and amplification was detected from DNA extracted from all isolates with a mean $C_T$ of 17 (SD 1.7).

### 6.2.7 **Summary of the performance of the real-time PCR assays**

The real-time PCR assays used for this study are shown in Table 6.1. Where possible previously published assays were chosen, however it was necessary to design novel assays for the detection of the *E. coli* O157:H7 intimin (*eaeγ*) gene, the *S. enterica* C-glycosyltransferase (*iroB*) gene and the *S. aureus* thermonuclease (*nuc*) gene. Validation of the *L. monocytogenes hlyA* real-time PCR can be found in section 3.2.Design of the green fluorescent protein gene (*gfp*) PCR can be found in section 4.2.1. All the oligonucleotide and probe positions for each gene target are shown in Figure 8.2 of Appendix 1.

**Table 6.1 Real-time PCR assays for the detection of foodborne pathogens used in this study.**

| Organism | Target | Acronym | Amplicon size (bp) | GeneBank accession number | Reference |
|---|---|---|---|---|---|
| *C. coli* | Periplasmic binding protein gene | *ceuE* | 103 | X88849 | Best *et al.* (2003) |
| *C. jejuni* | Membrane associated protein A gene | *mapA* | 96 | X80135 | Best *et al.* (2003) |
| *C. perfringens* | Alpha toxin gene | *cpa* | 103 | M24904 | Amar *et al.* (2005) |
| *C. perfringens* | Enterotoxin gene | *cpe* | 103 | M98037 | Amar *et al.* (2005) |
| *E. coli* O157:H7 | Intimin gamma | *eaeγ* | 142 | AF081182 | This study |
| *E. coli* GFP | Green fluorescent protein gene | *gfp* | 77 | M62654 | This study |
| *L. monocytogenes* | Haemolysin gene | *hlyA* | 113 | AY174657 | Nogva *et al.* (2000) |
| *S. aureus* | Thermonuclease gene | *nuc* | 113 | BX571856 | This study |
| *S. enterica* | C-glycosyltransferase | *iroB* | 91 | U62129 | This study |

Bp = base pairs

The PCR assays were evaluated in terms of the lowest level of detection, PCR efficiency, inclusivity and exclusivity. A summary of the findings is given in Table 6.2. The PCR assays ranged from $10^4$ to one genome copy(s) per PCR. The inclusivity and exclusivity of the PCR assays indicated that they there specific for their target and therefore unlikely to yield false positive or negative results due to nonspecific amplification. The PCR all had efficiencies of 93% or greater indicating as required for sensitive assays.

**Table 6.2 The lowest levels of detection and efficiency of 5' nuclease real-time PCR assays used in this study.**

| Organism | Gene target | Lowest level of detection | | PCR Efficiency | Inclusivity | | Exclusivity |
|---|---|---|---|---|---|---|---|
| | | CFU per PCR | Genome copies per PCR | | number detected/ number tested | Mean $C_T$ (SD) | |
| *C. coli* | *ceuE* | n/t | 1 | 95 % | 100 % (17/17) | 14 (2.61) | n/t |
| *C. jejuni* | *mapA* | n/t | 3375 | 95 % | 100 % (23/23) | 17 (4.44) | n/t |
| *C. perfringens* | *cpa* | n/t | 341 | 96 % | 100 % (39/39) | 17 (1.70) | n/t |
| *C. perfringens* | *cpe* | n/t | 341 | 96 % | 100% (33/33) | 17 (1.70) | n/t |
| *L. monocytogenes* | *hlyA* | 22 | 124 | 95 % | 100% (61/61) | 17 (1.44) | 100% |
| *E. coli* O157:H7 | *eaeγ* | 15 | 17 | 100 % | 100 % (40/40) | 20 (3.33) | 100% |
| *S. aureus* | *nuc* | n/t | 10 | 100 % | 100 % (107/107) | 18 (2.64) | 100% |
| *S. enterica* | *iroB* | n/t | 70 | 93 % | 100% (86/86) | 13 (3.00) | 100% |
| *E. coli* GFP | *gfp* | 0.5 | 10 | 102 % | n/t | n/t | 100% |

$C_T$ = Cycle threshold; SD = Standard deviation; n/t = not tested.

## 6.3 Results: Nested and hemi-nested PCR assays

The real-time PCR assays were shown to be specific and efficient, but lacked the very low levels sensitivity especially for the detection *C. jejuni, C. perfringens* and *L. monocytogenes*, which was required for the detection of these pathogens in food samples. This was demonstrated for *L. monocytogenes* in section 3.3.6, when the hlyA assay was applied to complex foods the assay lost one-log level of sensitivity. To increase the sensitivity of the PCR assays a nested or hemi-nested phase prior to the real-time PCR was developed.

### 6.3.1 **Development of a nested real-time PCR for the amplification of *L. monocytogenes hlyA* gene.**

The hlyA PCR for the detection of *L. monocytogenes* (sections 3.2.1- 3.2.3) had lower level of detection of 22 genome copies per PCR. However, when the sensitivity of the assay was determined PCR with whole cells rather than quantified DNA the lowest level of detection decreased to $3.44 \times 10^3$ CFU $ml^{-1}$.

A nested oligonucleotide set was designed as primary phase to amplify *L. monocytogenes hlyA* gene prior to real-time PCR amplification (section 2.9.10). The oligonucleotides termed hlyAOF and hlyAOR were designed to amplify a 157 bp fragment encompassing the region previously amplified with oligonucleotides hlyAF and hlyAR. The specificity of the oligonucleotide sequences were confirmed by BLASTN search (section 2.11.4). A PCR was performed with *L. monocytogenes* NCTC 12427 DNA and the new oligonucleotides (section 2.9.12) that resulted in a PCR product of the expected size as shown in Figure 6.5.

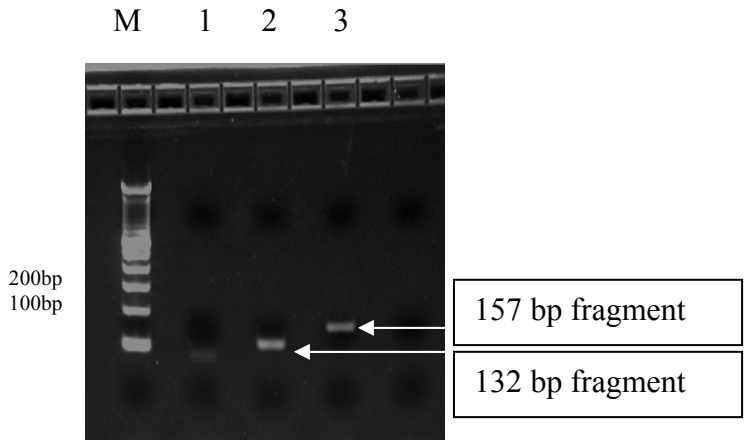



**Figure 6.5 Detection of *L. monocytogenes* NCTC 12427 *hlyA* gene by conventional PCR**.

Amplicons were analysed on a 2% E-gel (Invitrogen). Lane M 100bp ladder; Lane 1 negative control; Lane 2 *hlyA* gene fragment amplified using hlyAF and R oligonucleotides (second phase PCR); Lane 3 *hlyA* gene fragment amplified using hlyAOF and OR oligonucleotides (first phase PCR).

The specificity of the nested PCR assay (section 2.9.12) was tested using DNA extracted from 20 Gram positive and negative bacteria that occur in food shown in Table 6.3. A positive control of *L. monocytogenes* NCTC 12427 DNA (diluted to approximately 3.05 x $10^3$ CFU per PCR reaction) and a non-template control were used per experiment. The assay was found to be 100% specific, with no amplification observed with DNA extracted from any of the bacterial pathogens, except with the two *L. monocytogenes* strains.

A ten-fold decimal dilution of *L. monocytogenes* DNA was subjected to the first phase PCR using hlyAOF and hlyAOR oligonucleotides and the resulting amplicons were then amplified in the second phase that consisted of the real-time PCR assay using oligonucleotides hlyAF and hlyAR (section 2.9.12). *L. monocytogenes* amplification was detected in all samples except in the most concentrated *L. monocytogenes* DNA sample. This DNA sample was diluted ten-fold in nuclease-free water and re-applied to the assay resulting in a strong amplification ($C_T$ 4). This suggests that the negative result was due to inhibition of the PCR. The experiment was repeated using an internal amplification control (described in sections 3.2.4 and 3.2.5). The internal

control was not amplified which indicated inhibition. The inhibition was overcome by dilution of the amplicons after the first phase of PCR. In subsequent nested *L. monocytogenes* PCR assays, the phase one amplicons were diluted ten-fold in nuclease-free water.

**Table 6.3 Specificity of the 5' nuclease nested PCR assay for the detection of *L. monocytogenes hlyA* gene applied to a range of bacterial strains.**

| Bacteria | NCTC strain number | *L. monocytogenes hlyA* PCR result |
|---|---|---|
| *Aeromonas hydrophila* | NCTC 8049 | Not detected ($C_T \geq 40$) |
| *Bacillus cereus* | NCTC 7464 | Not detected ($C_T \geq 40$) |
| *Bacillus subtilis* | NCTC 10400 | Not detected ($C_T \geq 40$) |
| *Campylobacter coli* | NCTC 206 | Not detected ($C_T \geq 40$) |
| *Campylobacter jejuni* | NCTC 207 | Not detected ($C_T \geq 40$) |
| *Clostridium perfringens* | NCTC 8359 | Not detected ($C_T \geq 40$) |
| *Enterococcus faecalis* | NCTC 775 | Not detected ($C_T \geq 40$) |
| *Escherichia coli* | NCTC 9001 | Not detected ($C_T \geq 40$) |
| *Enterococcus durans* | NCTC 662 | Not detected ($C_T \geq 40$) |
| ***Listeria monocytogenes*** | **NCTC 11994** | **Detected ($C_T$ 18)** |
| ***Listeria monocytogenes*** | **NCTC 12427** | **Detected ($C_T$ 18)** |
| *Listeria innocua* | NCTC 11288 | Not detected ($C_T \geq 40$) |
| *Listeria ivanovii* | NCTC 11846 | Not detected ($C_T \geq 40$) |
| *Listeria welshimeri* | NCTC 11857 | Not detected ($C_T \geq 40$) |
| *Listeria seeligeri* | NCTC 11856 | Not detected ($C_T \geq 40$) |
| *Proteus rettgeri* | NCTC 7475 | Not detected ($C_T \geq 40$) |
| *Pseudomonas aeruginosa* | NCTC 10662 | Not detected ($C_T \geq 40$) |
| *Salmonella enterica* | NCTC 4840 | Not detected ($C_T \geq 40$) |
| *Staphylococcus aureus* | NCTC 6571 | Not detected ($C_T \geq 40$) |
| *Staphylococcus epidermidis* | NCTC 11047 | Not detected ($C_T \geq 40$) |
| *Vibrio furnissi* | NCTC 11218 | Not detected ($C_T \geq 40$) |
| *Vibrio parahaemolyticus* | NCTC 10885 | Not detected ($C_T \geq 40$) |

$C_T$ = cycle threshold; Samples positive for the target gene are shown in bold.

To compare the sensitivity of the nested assay (section 2.9.12) to the single-phase real-time assay (section 2.9.7), a ten-fold serial dilution of *L. monocytogenes* DNA was amplified in each assay. Standard curves of mean $C_T$ values were plotted and a linear relationship was obtained. The efficiency

of the nested *L. monocytogenes* PCR was y = -3.24 compared to the original *L. monocytogenes* PCR of y = -3.53, and the sensitivities are presented in Table 6.4.

**Table 6.4 Comparison of the PCR efficiency and sensitivity for the original and nested *L. monocytogenes hlyA* PCR assays**.

| PCR efficiency and sensitivity | *hlyA* PCR | Nested *hlyA* PCR |
|---|---|---|
| Slope of the Curve (y) | –3.53 | -3.25 |
| Coefficient of determination ($R^2$) | 0.996 | 0.962 |
| Sensitivity (CFU per PCR) | 1100 | 1.1 |
| Sensitivity (genome copies per PCR) | 4500 | 4.5 |

There was a loss of linearity for the nested assay ($R^2 = 0.962$) because the first phase amplifies DNA in an end-point PCR so the amplification was not exponential through out the complete assay. The amplicons were diluted before amplification and PCR linearity was determined from $C_T$ values produced in the second phase of PCR. The sensitivity of the nested assay was equal to 1.1 CFU or 4.5 genome copies per PCR (2.2 x 10 $CFUml^{-1}$), compared to 4500 genome copies per PCR for the un-nested assay.

The 95% Confidence intervals (section 2.10.3) were calculated to determine an upper $C_T$ limit for the *hlyA* assay. A standard curve of mean $C_T$ value against CFU per PCR with upper and lower 95% confidence limits was plotted. The upper and lower 95% confidence interval was equal to $p = < 0.05$ up to $C_T$ 35. This was therefore set as the upper cut-off $C_T$ value for the assay. An equivocal result was defined as one having a $C_T$ value of 36 to 40. With the cut off value applied, the efficiency of the amplification was y = -3.61 and linearity was improved to $R^2 = 0.999$. Positive result equalled an amplification of $C_T$ of 35 or below, an equivocal result equalled a $C_T$ value of 36 to 40 and undetected equalled a $C_T$ >40 when tested in the *hlyA* PCR assay.

### 6.3.2 **Application of the *L. monocytogenes* nested PCR for the detection of the bacterium in clinical samples**

DNA was extracted from seventeen serum and CSF samples collected from patients with culture confirmed (numbered one to nine) or suspected listeriosis (numbered 10 to 17) (sections 2.5.1 and 2.5.3). The extracts were subjected to the standard (section 2.9.7) and nested *hlyA* PCR assays (2.9.12) as shown in Table 6.5.

**Table 6.5 Application of nested and standard real-time PCR for the detection of *L. monocytogenes hlyA* gene as applied to cerebral spinal fluid and serum samples.**

| Sample number | Culture details or clinical details suggestive of *Listeria monocytogenes* infection | *hlyA* PCR | | Nested *hlyA* PCR | |
|---|---|---|---|---|---|
| | | Mean $C_T$ | Interpretation | Mean $C_T$ | Interpretation |
| 1 | ***L. monocytogenes* cultured** | 36 | Equivocal | 21 | Detected |
| 2 | | 33 | Detected | 20 | Detected |
| 3 | | 36 | Equivocal | 21 | Detected |
| 4 | | 36 | Equivocal | 20 | Detected |
| 5 | | 35 | Detected | 20 | Detected |
| 6 | | 36 | Equivocal | 18 | Detected |
| 7 | | 35 | Detected | 16 | Detected |
| 8 | | 35 | Detected | 19 | Detected |
| 9 | | 39 | Equivocal | >40 | Not detected |
| Negative control | | >40 | Not detected | >40 | Not detected |
| **Samples where *L. monocytogenes* was not previously cultured** | | | | | |
| 10 | Miscarriage, gastroenteritis | 30 | Detected | 11 | Detected |
| 11 | Meningitis | 39 | Equivocal | 21 | Detected |
| 12 | Miscarriage | 37 | Equivocal | 19 | Detected |
| 13 | Meningitis | 38 | Equivocal | 18 | Detected |
| 14 | Meningitis | 38 | Equivocal | 19 | Detected |
| 15 | Unexplained miscarriage | 29 | Detected | 10 | Detected |
| 16 | Pregnant proteinuria | 43 | Not detected | >40 | Not detected |
| 17 | Gastroenteritis and intrauterine death. | >40 | Not detected | 24 | Detected |
| Negative control | | >40 | Not detected | >40 | Not detected |

$C_T$ = cycle threshold. Positive resulted from $\leq C_T$ 35, an equivocal result equalled $C_T$ value of 36 to 40 and negative $C_T$ >40 ($C_T$ >40) when tested in the *hlyA* PCR assay.

*L. monocytogenes hlyA* gene was detected in samples 2, 5, 7, 8, 10 and 15 by both nested and un-nested PCR. *L. monocytogenes hlyA* gene was not detected in samples 16 and 17 un-nested PCR, however, in subsequent testing with the nested, PCR *L. monocytogenes hlyA* was detected in sample 17 ($C_T$ 24). Samples 1, 3, 4, 6, 9, 11-14 were found equivocal by the un-nested PCR, in 8/9 of these samples *L. monocytogenes hlyA* was detected using the nested assay. In 1/9 samples (sample 9) *L. monocytogenes hlyA* was not detected by nested PCR, however a $C_T$ 39 was obtained in the real-time assay that indicates an increase in florescence in the last cycle of the assay. At this point in the reaction a slight increase in florescence can be due to a non-specific reaction, hence the use of equivocal result, which was not confirmed using the more sensitive nested PCR. The sample was tested in duplicate in the assay and was not detected in either sample. *L. monocytogenes* had been cultured from this sample, however it may have been present at a level below the detection rate of the PCR (22 $CFUml^{-1}$).

### 6.3.3 **Development of hemi-nested conventional PCR**

To increase the sensitivity of all the real-time assays described previously (sections 6.2.1-6.2.9 and gfp in chapter 4) oligonucleotides for a first phase hemi-nested PCR were designed for each target gene (section 2.9.10). Table 6.6 lists the NCTC organism, target gene, and expected amplicon size that would result from the first and second phase PCR. The assays were designed with a reverse external oligonucleotide that was paired with the existing forward oligonucleotide.

**Table 6.6 Hemi-nested conventional PCR for detection of foodborne pathogenic bacteria used in this study.**

| Organism | NCTC | Target gene | Phase one amplicon size (bp) | Phase two amplicon size (bp) |
|---|---|---|---|---|
| [1]*E. coli* O157 | 12900 | *eaeγ* | 265 | 142 |
| *C. jejuni* | 11168 | *mapA* | 257 | 96 |
| [1]*S. aureus* | 6571 | *nuc* | 243 | 113 |
| *C. coli* | 12110 | *ceuE* | 231 | 103 |
| *C. perfringens* | 8239 | *cpe* | 181 | 103 |
| *L. monocytogenes* | 12427 | *hlyA* | 132 | 113 |
| *S. enterica* | 13349 | *iroB* | 139 | 91 |
| *C. perfringens* | 8239 | *cpa* | 132 | 103 |
| *E. coli* GFP | [2]n/a | *gfp* | 122 | 77 |

[1] Not shown on gel Figure 6.5. [2]*E. coli* GFP strain construction in this study (chapter 4)

The hemi-nested first phase PCR was applied to DNA extracted from the NCTC cultures of target organisms except *E. coli* GFP in monoplex reactions (section 2.9.11). The first phase amplicons were observed after gel electrophoresis (section 2.8.7) as shown in Figure 6.6, and were concordant sizes to those predicted (Table 6.6). The amplification of the *gfp* gene in first phase of the hemi-nested PCR for *E. coli* GFP is shown in section 4.2.1. No amplicon was detected for the *E. coli eaeγ* and *S. aureus nuc* assays. The PCR assays were repeated and amplicons of the expected size were observed after gel electrophoresis (data not shown).

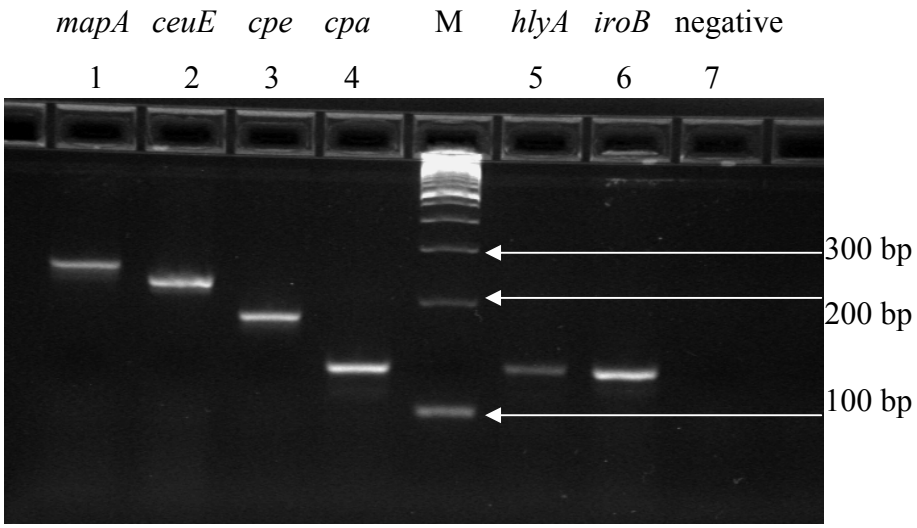



**Figure 6.6 Detection of gene targets by first phase hemi-nested monoplex PCR.**

Lane 1 *C. jejuni* (*mapA*), Lane 2 *C. coli* (*ceuE*), Lane 3 *C. perfringens* (*cpe*), Lane 4 *C. perfringens* (*cpa*), Lane M 100bp ladder, Lane 5 *L. monocytogenes* (*hlyA*), Lane 6 *S. enterica* (*iroB*), Lane 7 negative control. DNA amplified from target pathogens with F and RX oligonucleotides. Amplicons run on a 4 % agarose gel. Gene target given in parenthesis.

The first phase PCR amplicons were diluted ten-fold and amplified using the F and R oligonucleotides and pathogen specific conventional PCR (section 2.9.11) The amplicons were observed after gel electrophoresis (section 2.8.7) as shown in Figure 6.7, and were concordant sizes to those predicted (Table 6.6). An amplicon was not generated for the *E. coli* GFP (Lane 8), however the DNA extraction and hemi-PCR was repeated and the amplicon observed (Figure 6.8)

Sequencing the amplicons resulting from the hemi-nested PCR confirmed that the oligonucleotides used in both phases specifically amplified the correct fragment of the gene (section 2.11). Chromatograms from sequencing reactions are not shown. However, the probe and oligonucleotide binding sites for each target gene are shown in Figure 8.1 of Appendix 1. The next step was to run the hemi-nested PCR was as a multiplex reaction. The hemi-nested PCR was designed to produce small amplicons of similar sizes (see Table 6.6) to

reduce potential preferential amplification of the small amplicons and reduce the loss of sensitivity often observed when oligonucleotide pairs are multiplexed. An alternative method of amplicon detection was required and suspension microarray was therefore investigated

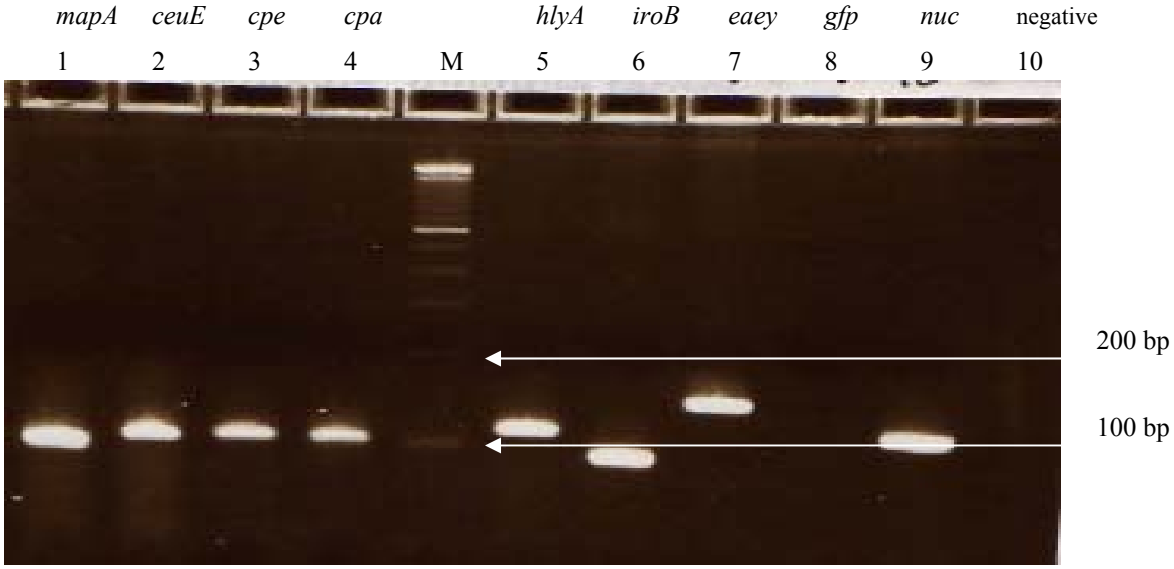



**Figure 6.7 Amplicons produced from hemi-nested monoplex PCR**

Lane 1 *C. jejuni* (*mapA*), lane 2 *C. coli* (*ceuE)*, lane 3 *C. perfringens* (*cpe)*, lane 4 *C. perfringens* (*cpa)*, lane M 100 bp ladder, lane 5 *L. monocytogenes* (*hlyA)*, lane 6 *S. enterica* (*iroB*), lane 7 *E. coli* O157:H7 (eaeγ) lane 8 *E.coli*-GFP (*gfp)*, lane 9 *S. aureus* (*nuc*), lane 10 negative control (nuclease–free water). Amplicons run on a 4% E-gel. Gene target given in parenthesis.

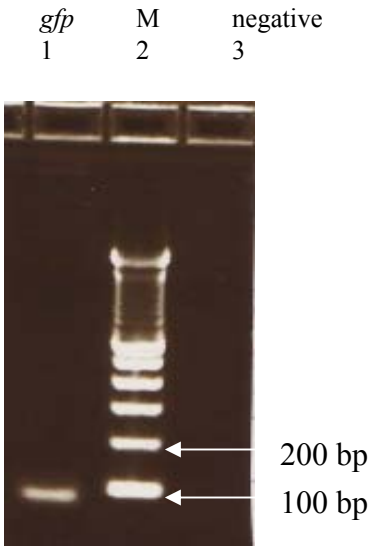



**Figure 6.8 GFP amplicon produced from hemi-nested PCR**

Lane 1 *gfp* gene target, lane 2 100 bp marker, lane 3 (negative). Amplicons run on a 4% E-gel.

## 6.4 Results: Multiplex PCR and Bio-plex detection of amplicons

To produce an assay that could rapidly and screen food samples simultaneously for multiple foodborne bacterial pathogens the hemi-nested PCR reactions were multiplexed. To detect the amplicons produced from this assay Bio-plex, a suspension bead array was investigated. All PCR oligonucleotides (listed in Table 2.6), except for those used for 16S rDNA PCR as this assays was not included in the multiplex PCR, were screened for potential cross-reactivity, formation of hairpin structures or dimerisation using Autodimer described by Vallone and Butler (2004). A cross-reaction was found between oligonucleotides gfpRX and eaeγR, and dimerisations for gfpRX and nucR.

### 6.4.1 **Design of the probes for Bio-plex suspension bead array assay.**

The probes suspension bead array assay were designed as described by the manufacturer to detect all nine gene fragment that were: *C. jejuni* (*mapA*), *C. coli* (*ceuE)*, *C. perfringens* (*cpe)*, *C. perfringens* (*cpa)*, *L. monocytogenes* (*hlyA)*, *S. enterica* (*iroB*), *E. coli* O157:H7 (eaeγ), *E. coli*-GFP (*gfp)*, *S. aureus* (*nuc*). The probes consisted of capture oligonucleotides that were complementary to the target genes (section 2.12.1), which were bound to a set of carboxylated microspheres (section 2.12.2). Each set of microspheres contained a unique address that was associated only with the capture oligonucleotides complementary to one specific gene target. The combined oligonucleotide and microspheres formed the probe used to identify amplicons.

### 6.4.2 **Generation of amplicons for detection in the Bio-plex optimisation experiments**

DNA from the target pathogens, *C. coli, C. jejuni, C. perfringens, E. coli* O157:H7, *E. coli* GFP, *L. monocytogenes, S. aureus and S. enterica,* were amplified in monoplex conventional PCR assay with a biotinylated oligonucleotide (section 2.9.11). Successful amplification was confirmed by

visualisation of amplicons after gel electrophoresis and ethidium bromide staining (section 2.8.7) as shown in Figures 6.6 and 6.7 (equivalent representation of the gel). The concentration of DNA in the samples was calculated using Bionumerics software to compare the band intensity of the ethidium bromide stained amplicons to the bands from the quantification ladder (section 2.5.12).

### 6.4.3 **Bio-plex suspension bead array detection of amplicons using manufacturer's recommendations**

The amplicons (45ng) were initially hybridised to capture probes using the manufacture's conditions (1 x hybridisation buffer TMAC tetramethyl-ammonium chloride [TMAC] and hybridisation temperature of 55 $^{o}$C) (section 2.12.3). Both monoplex (single probe type and amplicon type per well) and multiplex conditions (all probe types and all amplicon types per well) were tested then detected using the Bio-plex (section 2.12.4).

Detection of the probes bound to the amplicons was measured as mean fluorescent intensity (MFI), which is a measure of the mean fluorescence of up to 120 beads that have bound to the complementary target after the background signal has been subtracted. The background signal is measured from designated well, which contains beads but no target amplicons. A result was considered positive if the MFI was three times greater than the background signal. Ideally, the amplicon and probe hybridisation reactions would result in equal MFI for all target amplicons in a reaction that contained equal amounts of amplicon and probe for each target.

*L. monocytogenes* (*hlyA*), *C. jejuni* (*mapA*), *C. coli* (*ceuE*), *S. enterica* (*iroB*) and *S. aureus* (*nuc*) amplicons were detected from single target amplicons and pools of beads only containing probes specific for the target amplicon (shown in pink in Figure 6.9). When a pooled sample of amplicons and specific beads were applied to the Bio-plex assay *L. monocytogenes* (hlyA), *C. jejuni* (mapA), *C. coli* (ceuE) and *S. enterica* (iroB) were detected (shown in blue in Figure 6.9). When a pooled sample of amplicons and pooled beads were applied to the Bio-plex assay *L. monocytogenes* (hlyA), *C. jejuni* (mapA), *C.*

*coli* (ceuE), *S. enterica* (iroB), *E. coli* GFP (gfp) and *S. aureus* (nuc) were detected (shown in yellow in Figure 6.9).

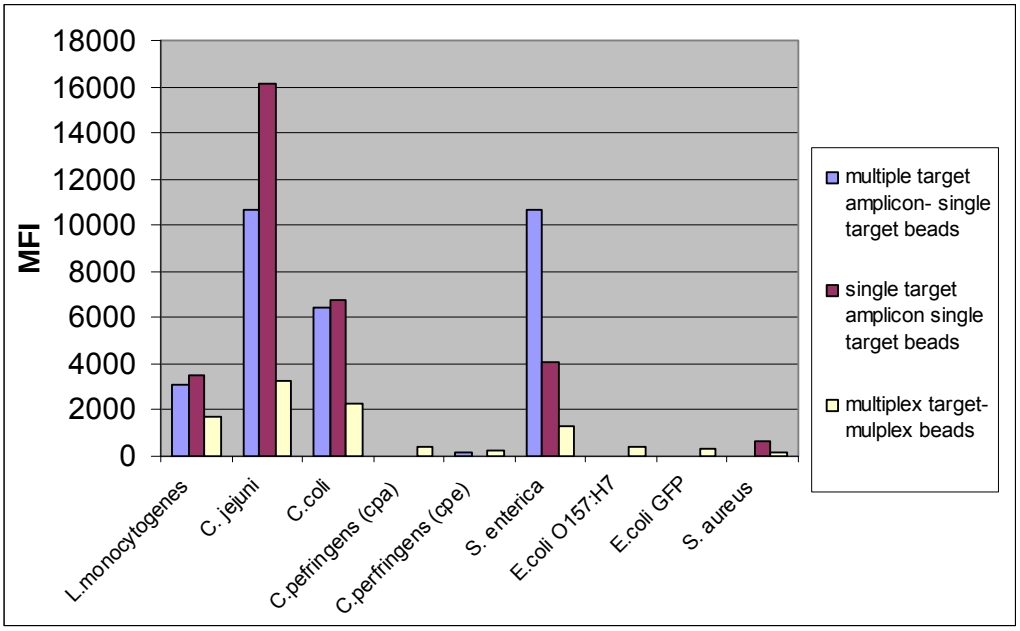



**Figure 6.9 Comparison of Bio-plex detection of multiplex and monoplex produced amplicons using pooled and un-pooled capture probes.**

Detection of amplicons from multiplex reaction and target specific beads, monoplex reaction and single target beads, and multiplex reaction and multiplex beads for all nine target reactions. A positive reaction was determined from the Mean fluorescence intensity (MFI).

However, MFI was reduced in the multiplex assay as compared to the monoplex reactions. *C. perfringens* (*cpa* and *cpe*), *E. coli* GFP (*gfp*) and *E. coli* O157:H7 (*eaeγ*) gene targets were not detected in any assay, the slight MFI signal was not sufficient (i.e. at least three times higher then the background) to be considered a positive result. In addition, the amplicons did not result in comparable MFI's therefore the assay required optimisation.

### 6.4.4 **Optimisation of Bio-plex probe hybridisation buffer concentration**

A concentration titration of the hybridisation buffer TMAC was performed. Amplicons (45ng) from all nine target genes were hybridised to capture probes (hybridisation temperature of 55 $^{o}$C) (section 2.12.3) and were detected using the Bio-plex (section 2.12.4). A TMAC concentration titration from 2.0M to 4.5M was applied to a pool of amplicon from all gene targets and applied to a pool of all the probes (Figure 6.10). The gene targets for the detection of *S. aureus* (*nuc*), *C. perfringens* (*cpa* and *cpe*) and *C. coli* (*ceuE*) were undetected. The gene targets for the detection of *E. coli* GFP (*gfp*), *S. enterica* (*iroB*), *C. coli* (*ceuE*), *E. coli* O157:H7 (*eaey*) and *L. monocytogenes* (*hlyA*), MFI increased with decreasing TMAC concentration. The concentration of the buffer affects probe to amplicon binding.

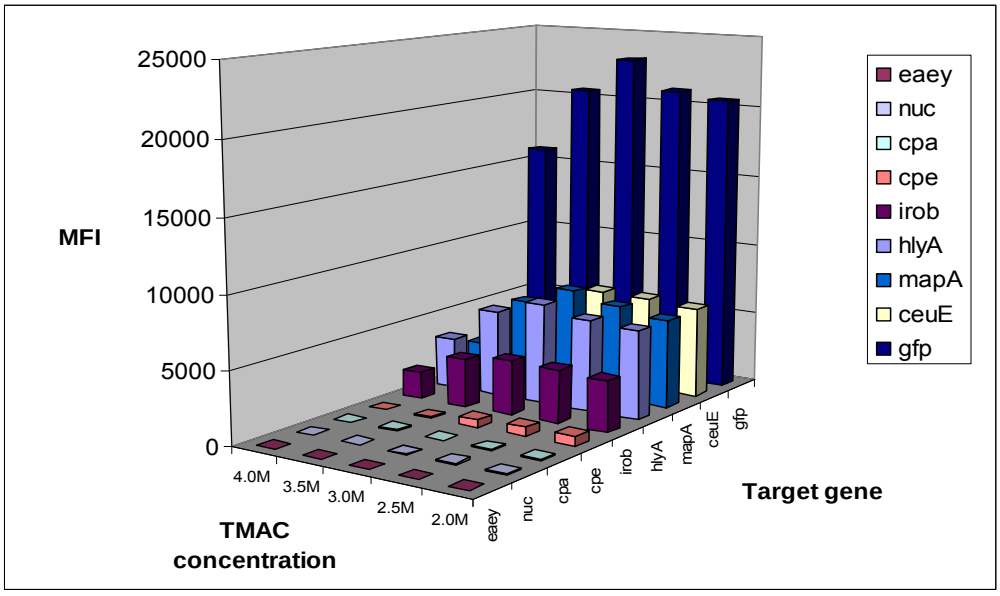



**Figure 6.10 The effect of varying tetramethylammonium chloride concentration on mean fluorescence intensity for detecting multiple amplicons simultaneously in a Bio-plex assay.**

TMAC = tetramethylammonium chloride**.** Detection of amplicons from multiplex reaction and multiplex beads for all nine target reactions. A positive reaction was determined from the Mean fluorescence intensity (MFI). Organism and gene target: *S. aureus* (*nuc*), *C. perfringens* (*cpa* and *cpe*), *C. coli* (*ceuE*), *E. coli* GFP (*gfp*), *S. enterica* (*iroB*), *C. coli* (*ceuE*), *E. coli* O157:H7 (*eaey*) and *L. monocytogenes* (*hlyA*).

The stringency of binding is reduced as the concentration of TMAC is reduced. At TMAC concentrations of 3.0M to 4.5M the MFI was not appreciably increased. Reducing the TMAC concentration reduced the background signal detected from the negative control samples and resulted in an increased MFI. A TMAC concentration of 3.0M gave a higher MFI than 2.0M or 2.5M for all gene targets that were detected, therefore 3.0M TMAC was selected as optimal.

### 6.4.5 **Optimisation of Bio-plex probe hybridisation temperature**

To improve the MFI for all targets and to detect the *C. perfringens* gene targets, the amplicon-probe hybridisation temperatures were optimised. A temperature gradient was applied to each target amplicon and respective probe over a temperature range of 40 $^{o}$C to 55 $^{o}$C. Amplicons (45ng) from all nine target genes were hybridised to capture probes using 3M TMAC (section 2.12.3) and were detected using the Bio-plex (section 2.12.4). MFI increased with decreasing hybridisation temperature for the majority of the gene targets (Figure 6.11).

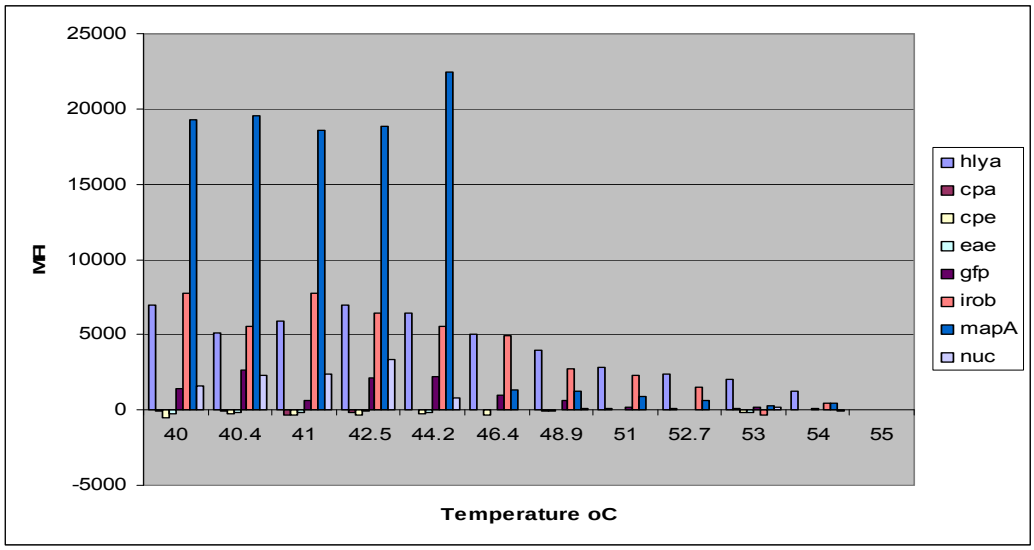



**Figure 6.11 Effect of varying the hybridisation temperature on mean fluorescence intensity for detecting multiple amplicons simultaneously in a Bio-plex assay.**

A positive reaction was determined from the Mean fluorescence intensity (MFI). Organism and gene target: *S. aureus* (*nuc*), *C. perfringens* (*cpa* and *cpe*) *E. coli* GFP (*gfp*), *S. enterica* (*iroB*), *C. coli* (*ceuE*), *E. coli* O157:H7 (*eaey*) and *L. monocytogenes* (*hlyA*).

The hybridisation temperature that resulted in the highest MFI for each target detected was: *E. coli* GFP (*gfp*) 40.4 $^{o}$C, *S. enterica* (*iroB*) 40-41 $^{o}$C, *S. aureus* (*nuc*) 42.5 $^{o}$C, *L. monocytogenes* (*hlyA*), 42.5 $^{o}$C and *C. jejuni* (*mapA*), 44.2 $^{o}$C. Gene targets *C. perfringens* (*cpa* and *cpe*), *C. coli* (*ceuE*) and *E. coli* O157:H7 (*eaey*) were undetected.

### 6.4.6 Redesign of *C. perfringens* (cpa and cpe) and *E. coli* O157:H7 (eaey) Bio-plex probes

The probes for *C. perfringens* (cpa and cpe), and *E. coli* O157:H7 (*eaey*) assays were redesigned (section 2.12.1) and tested in monoplex using 3M TMAC and hybridisation of 44 $^{o}$C (sections 2.12.3 and 2.12.4). Amplicons (45ng) from all nine target genes were hybridised to capture probes using 3M TMAC (section 2.12.3) and were detected using the Bio-plex (section 2.12.4). The all the gene targets except *nuc* and *cpe* were detected (Figure 6.12). The redesigned *eaeγ* resulted in MFI = 9504 and *cpa* MFI = 2146, which was successful. The multiplex-suspension bead array was abandoned, as not all the targets could be successfully detected and further optimisation was outside of the time limits of this study.

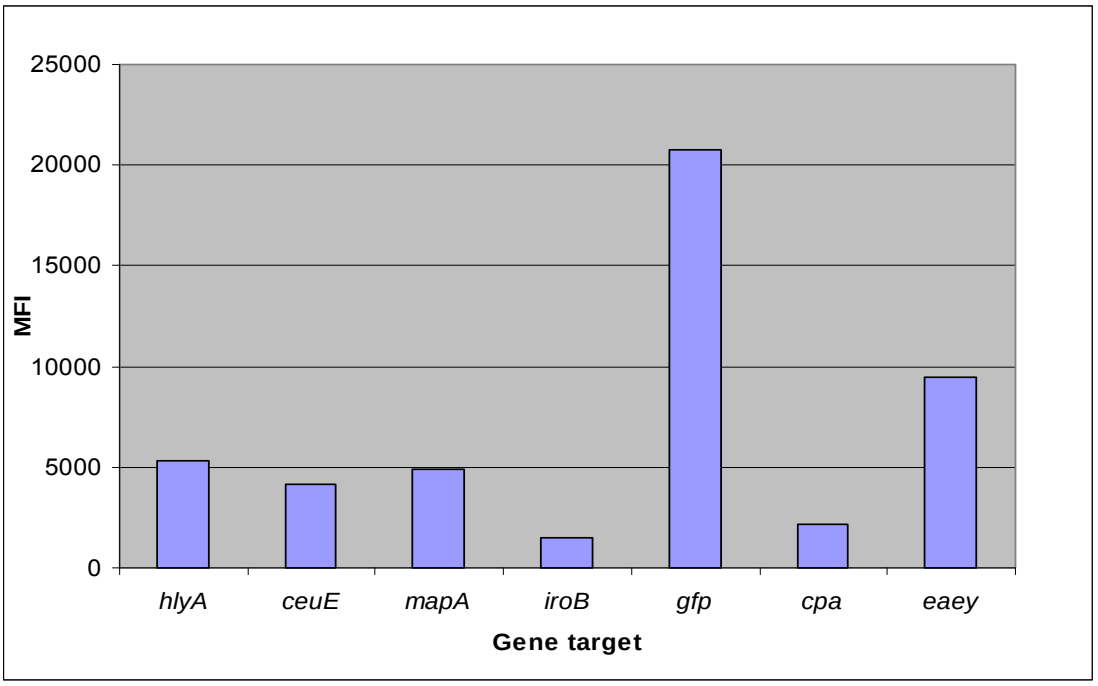



**Figure 6.12 Bio-plex detection of a pool of multiple gene targets using optimised probe hybridisation conditions.**

A positive reaction was determined from the Mean fluorescence intensity (MFI). Organism and gene target: *C. perfringens* (*cpa*), *C. coli* (*ceuE*), *E. coli* GFP (*gfp*), *S. enterica* (*iroB*), *C. coli* (*ceuE*), *E. coli* O157:H7 (*eaey*) and *L. monocytogenes* (*hlyA*).

## 6.5 Discussion

The aim of the work presented in this chapter was to design a multiplex PCR for the simultaneous and sensitive detection of eight target bacteria. First, three novel real-time PCR assays were designed for the detection of *E. coli* O157:H7, *S. enterica* and *S. aureus.* A hemi-nested phase of PCR was designed for each target pathogen. A nested PCR assay was designed and applied for the sensitive detection of *L. monocytogenes* in serum and CSF samples. Finally, the second phase PCR assays were multiplexed and the detection of the amplicons by suspension bead array was investigated.

### 6.5.1 Selection and design of PCR assays for the detection foodborne pathogens

Published PCR assays were selected or designed for the detection of the most significant foodborne bacterial pathogens, namely: *C. jejuni* and *coli*; *C. perfringens*; *E. coli* O157:H7; *S. enterica, L. monocytogenes* and *S. aureus*. Previously published assays were selected for the detection of *L. monocytogenes, C. perfringens*, *C. jejuni* and *coli* as these PCRs had been used extensively within the Health Protection Agency to confirm the identity of isolates from food and clinical samples. Real-time PCR assays have been published for the detection of *E. coli* O157:H7 (Sharma, 2006) *S. enterica* (Hoorfar *et al.,* 2000; Liming and Bhagwat, 2004; Rodriguez-Lazaro *et al.,* 2003), and *S. aureus* (Hein *et al.,* 2005; Hein *et al.,* 2001), but use different PCR conditions than the assays currently used in this study. The selection of a gene target for detection of *E. coli* O157:H7, *S. enterica* and *S. aureus* was first required for development of PCR assays that could be subsequently used in a multiplex format.

The *C. perfringens* PCR assay targets the alpha toxin gene (*cpa*) that is present in all *C. perfringens* strains. The alpha toxin gene codes for a phospholipase C, which is a toxin with haemolytic, lethal dermonecrotic, vascular permeabilization and platelet aggregating properties (Titball, 1993). The alpha

toxin plays a role in gas gangrene and inflammatory responses in iletis and Crohns disease (Titball, 1993). However it is the *C. perfringens* enterotoxin gene (*cpe*) that produces a 35 kDa protein that causes foodborne diarrhoeal disease (Hauschild *et al.,* 1971). The PCR adopted in this study for the simultaneous detection of *C. perfringens cpe* and *cpa* genes was previously described by Amar *et al.* (2005). In the original study *C. perfringens* (*cpa* gene fragment) was detected in 52/53 (98%) of the samples also found culture positive for *C. perfringens. C. perfringens* (*cpe* gene fragment) was detected in 18/53 (34%), which were also found to be toxin positive by RPLA or ELISA and there have been no reports of false-positive amplifications. The specificity of the *C. perfringens* assay for identification of the organism was also confirmed using 253 culture confirmed isolates and the enterotoxin gene was also detected in 88 % of these samples (Grant *et al.,* 2008). In addition no false positives were detected when the assays were tested against 18 *Bacillus* spp., 6 *L. monocytogenes* and 22 *Clostridia* spp. (non- perfringens toxigenic type A) (Grant *et al.,* 2008).

In the study presented here, the assay was tested against 33 and *C. perfringens* 39 strains containing the *cpe* and *cpa* genes respectively and found to be specific with 100% inclusivity. Due to the exclusivity testing performed by Grant *et al.* (2008) and Amar *et al.* (2005) no further testing was done in this study. The lowest level of detection was not reported in the previous studies, but was measured for each gene independently resulting in 341 genome copies per PCR for both genes and both PCR assays were 96% efficient. Subsequent to the work in this thesis, a more sensitive real-time FRET (Fluorescence Resonance Energy Transfer) PCR for the detection of *C. perfringens cpe* gene fragment was published (dela Cruz *et al.,* 2006). This group applied their assay to MagNA Pure™ extracted DNA from food homogenates spiked with $10^4$ *C. perfringens* cells, but their assay did not contain an internal positive control.

Best *et al.* (2003) published the ceuE and mapA duplex assay for the speciation of *C. jejuni* and *C. coli* and this was selected for use in this study. When performed as a duplex, a detection limit of 20 CFU for both target was

reported by Best *et al.* (2003). The lowest level of detection of the ceuE assay, as used in this thesis, was one genome copy per PCR and the mapA PCR was 3375 genome copies per PCR. The decreased sensitivity of the mapA reaction may have been due to the assays being run with the IPC target. The ceuE assay was more sensitive in this study, which may have been due to the reporter dye being changed from VIC to FAM so that it could be duplexed with the IPC.

A PCR for the detection of *E. coli* O157:H7 was developed, which targeted the distal region of the gamma intimin gene. Due to the severe illness caused by VT producing strains of *E. coli*, if there is suspicion of the presence of it in a sample, then it must be handled at containment level three as advised by the Advisory Committee of Dangerous Pathogens (ACDP). In this work *E. coli* O157:H7 NCTC 12900 strain was selected as the control strain and used for the validation studies as it contained *eaeγ* gene fragment, but lacked the VT genes and so could be handled at containment level two.

The real-time eaeγ PCR was 100% efficient over a seven-log dilution range and had a limit of detection of 17 genome copies per PCR. The PCR assay was validated by testing it against 40 patient isolates and PCR confirmed *E. coli* O157:H7 and ten enteropathogenic *E. coli* control strains that were gamma intimin negative. Only one target gene was selected for the detection of *E. coli* O157:H7 and the assay could have been improved by the addition of PCRs targeting genes coding for the verocyto-toxin genes. However, if the *eaeγ* gene fragments were detected in a food product by this PCR, it would be recommended that the food was analysed using standard culture techniques (section 2.13.1). Culture of the organism can confirm the PCR result and provide an isolate of the *E. coli* O157:H7, which can be further typed, screened for the presence of VT genes or alternative virulence factors.

Initially, a real-time PCR targeting the *invA* gene specific for *Salmonella* was designed that would be compatible with the internal control strategies used in this study, however, this PCR did not produce a fluorescent signal. An alignment of multiple sequences of the *Salmonella invA* gene showed

numerous single nucleotide polymorphisms throughout the gene. A region of the gene that was conserved and contained sequences that could be amplified using TaqMan conditions (described in section 2.9.7) was not found so this gene target was abandoned. An alternative real-time PCR targeting the *S. enterica iroB* gene was developed. It was shown to be 93% efficient, specific and gave a low level of detection equal to 15 CFU per PCR. To the best of our knowledge, this is the only real-time PCR assay targeting the *iroB* gene specific to *S. enterica*

A real-time PCR was developed for the detection of *S. aureus* thermonuclease gene and it was shown to be 100% specific and efficient. Hein *et al.* (2001) developed a real-time quantitative PCR for the detection of *S. aureus* in cheese samples using a TaqMan probe. This assay could detect 30 genome copies per PCR, comparable to the 22 copies per PCR found with the nuc PCR described in this study. The assay described by Hein *et al.* (2001) had the advantage of being validated to quantify the numbers of *S. aureus* cells, however it did not include an internal control to monitor for false negative PCR results which was a requirement for this study. As with the *E. coli* O157:H7 assay, targeting only one gene in the *S. aureus* assay is not sufficient. Ideally, on detecting *S. aureus* in a food sample, the analyst should also attempt to detect enterotoxin in the food sample and detection of those entertoxin genes from the isolate and possible further epidemiological investigations if the food was associated with a food poisoning incident.

### 6.5.2 **Development and application of a nested PCR for the detection of *L. monocytogenes***

The *L. monocytogenes hlyA* real-time assay used in this study was validated as described in section 3.2.1-3.2.3. It was shown that the sensitivity of the assay was reduced one to two logs depending on matrices when applied directly to food samples. Nested PCR was developed to improve the sensitivity of the *hlyA* PCR. By developing a nested phase, the sensitivity of the PCR assay was increased by three logs to a lower level of detection of 22 $CFUml^{-1}$ compared

to the single-phase real-time PCR assay. The nested *L. monocytogenes* PCR performed in this study was more sensitive than the 200 CFU per ml limit detected by Jaton *et al.* (1992) who used a conventional nested PCR targeting a fragment of the invasion-associated protein gene (*iap*) specific to *L. monocytogenes* (Jaton *et al.,* 1992). Bäckman *et al.* (1999) reported a limit of detection of $10^5$ $CFUml^{-1}$ for *L. monocytogenes* using Dynal nucleic acid isolation with a hemi-nested PCR targeting 16S rDNA, which was also not as sensitive as the assay described in this study. The nested assay described in this chapter was more sensitive than the assays published by Jaton *et al.* (1992) and Backman *et al*. (1999), which may have been due to the second phase being performed on a real-time platform. The real-time PCR phase used fluorescent probes to detect the amplicons that are more sensitive than gel electrophoresis detection methods.

Although this study was specifically looking at the detection of pathogens in foods, identification of the same pathogens in clinical samples is essential to identify the organisms that have caused illness. Therefore, the PCR assays described in this study could potentially also be applied to clinical samples. *L. monocytogenes* is capable of causing severe and even fatal infections in certain high-risk patient groups including pregnant women and neonates. A definitive diagnosis of listeriosis depends upon culturing the organism from the blood or cerebral spinal fluid (CSF). However, low numbers of the bacterium may be present in both samples and problems with culturing this bacterium after commencement of antimicrobial therapy is well-recognised (Backman *et al.,* 1999). As part of this thesis, the nested assay was applied to clinical samples for the detection of *L. monocytogenes* to assess the assay's performance with these samples. Although only 17 samples were tested, the results of this work indicated that there was an increased detection of *L. monocytogenes hlyA* gene using the nested PCR when compared to the single-phase real-time *hlyA* assay.

### 6.5.3 **Multiplex detection of foodborne pathogens**

The sensitivity of PCR assays is often reduced when oligonucleotides are multiplexed due to competition. The sensitivity of *L. monocytogenes hlyA* PCR was improved by adding an additional phase of PCR to the assay and this approach was investigated for the multiplex to maintain or improve the sensitivity. A hemi-nested first phase was selected in preference to a nested phase, as only one additional oligonucleotide was required. By using a hemi-nested approach rather than nested, the potential cross-reactions between oligonucleotides was reduced as nine less oligonucleotide were required.

A hemi-nested multiplex PCR for the simultaneous detection of nine targets comprising of *C. perfringens* (*cpa* and *cpe*), *E. coli* GFP (*gfp*), *E. coli* O157:H7 (*eaey*), *L. monocytogenes* (*hlyA*), *C. jejuni* (*mapA*), *C. coli* (*ceuE*), *S. enterica* (*iroB*) and *S. aureus* (*nuc*) was developed. The assay was shown to detect the target genes in monoplex hemi-nested assays. The second phase amplicons were small ranging from 76- 142 bp in size and it was hoped that this would reduce the competition between oligonucleotide pairs and so reduce the potential for loss of sensitive of the individual assays when multiplexed. A disadvantage of this strategy was that the amplicons could not be easily differentiated using agarose gel separation. Also current real-time PCR systems described to date have been limited the detection of four targets in the same well due to the detection channels of the flurophores (Ishii *et al.,* 2007; Persson *et al.,* 2005). Therefore, a suspension bead array was investigated as an alternative method of detecting amplicons.

Probes were designed for each of the gene targets and attached to the bead then used to detect the amplicons. The hybridisation was optimised by altering TMAC concentrations and hybridisation temperature, however it was not possible to detect all the gene targets. *C. perfringens* (*cpa*) and *E. coli* O157:H7 (*eaeγ*) assays were redesigned and the associated gene targets were detected however *C. perfringens* (*cpe*) gene target was not. This multiplex PCR and suspension bead array detection assay has provided some promising

preliminary results. However, at present the assay would require considerable further optimisation and validation before it could be applied for the detection of bacterial pathogens in food samples.

### 6.6 Conclusion

The hyphothesis being tested in the work described in this chapter was can PCR assays can be designed or modified to be robust and reliable when applied to the detection of bacterial pathogens directly in food samples. Novel real-time PCR assays were designed for the detection of *E. coli* O157:H7, *S. enterica* and *S. aureus.* Nine hemi-nested PCR assays were designed for detection of, *mapA* (*C. jejuni*), *ceuE* (*C. coli*), *cpe* (*C. perfringens* enterotoxin), *cpa* (*C. perfringens* alpha-toxin), *hlyA* (*L. monocytogenes*), *iroB* (*S. enterica*), *eaeγ* (*E. coli* O157:H7), *nuc* (*S. aureus*) and *gfp* (*E.coli* GFP) gene fragments. Bio-plex suspension bead array was investigated as a method of simultaneously detecting amplicons generated from the multiplex PCR. It was possible to detect seven of the nine gene targets, *nuc* was lost during Bio-plex optimisation and the assay failed to detect *cpe* gene target. Despite the promising preliminary results from multiplex assay, at present the assay requires further optimisation before it could be applied to naturally-contaminated food samples. However, if optimised the assay has the potential to detect up to 120 targets simultaneously and this could allow the analyst to screen for further foodborne bacteria or further target genes such as verocyototoxin genes of *E. coli* or specific serotypes of *Salmonella.*

In conclusion, it was not possible to detect all the target pathogens using the multiplex PCR and Bio-plex detection assay. The PCR assays were not sufficiently sensitive to detect the pathogens in foods unless cultural enrichment was appplied. The real-time assays were 100% specific and rapid returning a result from DNA extraction to detection in less than four hours.

# CHAPTER 7

# OPTIMISATION AND APPLICATION OF REAL-TIME PCR ASSAYS FOR THE DETECTION OF BACTERIAL PATHOGENS IN NATURALLY-CONTAMINATED FOODS

## 7.1 Experimental rational

PCR detection of food pathogens directly in foods has been demonstrated as a rapid alternative technique for the detection of food pathogens, however several challenges have prevented the widespread use of this technology in food laboratories (Abubakar *et al.,* 2007). In this thesis the use of an automated, high through-put generic DNA extraction technique that is applicable to food samples (chapter 3), developed two internal positive controls to monitor for PCR inhibition to increase robustness (chapters three and four) have been developed and finally incorporated in a real-time PCR with internal controls to produce sensitive, specific and robust assays for the detection of foodborne pathogens in food samples has been investigated. The individual components of the assays were developed and evaluated using axenic laboratory control organisms or artificially contaminated food samples. However, before the methods can be used routinely by a food microbiology laboratory a validation, using naturally-contaminated samples, must be performed.

Method validation is used to demonstrate that a new method can generate results that are comparable or better than those obtained by the current reference method and confirms the specificity and reproducibility of the method (Malorny *et al.,* 2003b). Validation of a PCR method for detection of foodborne pathogens consists of comparing it to culture detection, the current reference method of foodborne pathogens using artificial and naturally-contaminated food samples and investigating how it behaves in an inter-laboratory study (Malorny *et al.,* 2003b). The robustness of a test is best challenged using data produced by diagnostic staff working in real-life situations (Hoorfar *et al.,* 2004b).

The hypothesis of this section of the thesis was, that if PCR assays can be designed or modified to be robust and reliable when applied to the detection of bacterial pathogens directly in food samples, then they should offer a rapid alternative to cultural detection in samples from naturally contaminated food

samples. The work reported in this chapter describes the optimisation and comparison the PCR assays incorporating the MagNA Pure™ DNA extraction, the IPC or lenticulated *E. coli* GFP process control, to test this hypothesis in naturally-contaminated food samples and compares these methods to culture-based methods.

The specific objectives were:

1) optimise PCR incorporating IPC and PCR assays incorporating process controls,

2) apply the PCR assays with IPC for the detection of *S. aureus* (*nuc* gene) and *C. perfringens,* (*cpa,* and *cpe* genes) and *L. monocytogenes* to naturally-contaminated foods,

3) apply the PCR assays with PC for the detection of *L. monocytogenes, C. coli, C. jejuni, E. coli* O157:H7 and *S. enterica* to naturally-contaminated food samples in a field trial,

4) apply the PCR assay for the detection of *S. enterica* to samples investigated as part of an egg contamination incident and samples from an outbreak of Salmonellosis at a nursing home, and

5) summarise the findings of the study.

## 7.2 Results: Optimisation PCR assays incorporating internal positive control or process control for detection of bacterial pathogens in naturally-contaminated foods

Real-time PCR assays were selected for the detection of foodborne bacterial pathogens in food samples. However, PCR assays can be inhibited when applied to food matrices and give false negative results. To monitor for PCR inhibition, two positive controls were selected. The first, an internal positive control (IPC) was used to monitor for inhibition solely in the PCR. The IPC was previously used in this study in the *L. monocytogenes* PCR assay that was applied to complex food samples (section 3.2.1 - 3.2.3). In this section the IPC was duplexed with the *S. aureus* (*nuc* gene) and *C. perfringens,* (*cpa,* and *cpe* genes) assays.

The second positive control was an internal process control (PC), which monitored inhibition in the complete assay from the extraction of DNA to detection of amplicons. The PC was developed in this study and described in Chapter 4. In this chapter, the PC was duplexed with the PCR assays for the detection of *L. monocytogenes*, *C. coli, C. jejuni, E. coli* O157:H7 and *S. enterica* and optimised for application to food samples.

For all the validation experiments, NCTC cultures were used as described in section 2.1.1. DNA was extracted using MagNa Pure (section 2.5.1 and 2.5.2) for all experiments in this section (i.e. 7.2).

### 7.2.1 Internal positive control (IPC) PCR assay optimisation for detection of foodborne bacterial pathogens

To monitor amplification inhibition, the real-time PCR assays for the detection of *S. aureus* (*nuc* gene) and *C. perfringens,* (*cpa,* and *cpe* genes) were duplexed with a commercially available internal positive control (IPC) reaction (section 2.9.8). The reactions were optimised to work as duplex PCR assays consisting of the bacterial target gene and the IPC. The optimal

concentration of IPC DNA was determined by applying the duplex assay to a ten-fold serial dilution of bacterial DNA and comparing this to the results obtained in monoplex reactions (Table 7.1).

**Table 7.1 Level of detection for re-optimised PCR assays when duplexed with the IPC internal control.**

| Target organism | Target gene | Amplification $C_T$ (SD) | | | |
|---|---|---|---|---|---|
| | | Monoplex[1] | Duplex[2] IPC DNA concentration | | |
| | | | Neat | $10^{-1}$ | $10^{-2}$ |
| *S. aureus* | *nuc* | 17 (0.02) | 17 (0.02) | 17 (0.01) | 17 (0.09) |
| | IPC | n/a | 26 (0.27) | 34 (0.87) | 40 (0) |
| *C. perfringens* | *cpa* | 20 (0.07) | 21 (0.06) | 21 (0.13) | 21 (0.24) |
| | IPC | n/a | 26 (0.16) | 33 (0.53) | 40 (0) |
| *C. perfringens* | *cpe* | 22 (0.11) | 22 (0.01) | 22 (0.09) | 22 (0.02) |
| | IPC | n/a | 25 (0.04) | 30 (.70) | 37 (0.06) |

[1]Monoplex = pathogen target gene only. [2]Duplex = Pathogen target gene and IPC target. SD= standard deviation. IPC = internal positive control.

The previous experiment was repeated using different concentrations of IPC in serial dilutions of target DNA to select the IPC concentration that could reliably be detected whilst not adversely affecting sensitivity in detecting the bacterial target gene. The IPC DNA was added to the reaction undiluted (as supplied in the kit). No loss of sensitivity was detected in the bacterial reaction compared to the monoplex assays and therefore no dilution was required.

In the optimised assay conditions, the IPC reaction produced $C_T$ values of 26, 26 and 25, when duplexed with the nuc, cpa and cpe, reactions respectively. The results of the IPC duplex assays were interpreted as positive, negative or inhibited for detection of the target gene depending on whether amplification was detected in each reaction (i.e. bacterial gene and IPC of the assay).

### 7.2.2 **Process control (PC) PCR assay optimisation for detection of foodborne bacterial pathogens**

The *E. coli* GFP and green fluorescent protein (gfp*)* PCR were developed as a process control (PC) for use in real-time detection of bacterial pathogens (chapter 4). Real-time PCR assays for the detection of the target pathogens *L. monocytogenes* (Nogva *et al.,* 2000); *C. coli, C. jejuni* (Best *et al.,* 2003); *E. coli* O157:H7 and *S. enterica* (Chapter 6) were selected to be duplexed with the process control assay. The assays used the same PCR reaction conditions as the gfp assay indicating they could be duplexed (section 2.9.7). The pathogen detection assays were duplexed with the PC assay and applied to DNA from the target pathogen in standard real-time PCR conditions (section 2.13.2). A log reduction in sensitivity was observed for each of the pathogen specific reactions when duplexed with the gfp reaction when compared to the pathogen PCR run as a monoplex (data not shown)

To investigate the loss of sensitivity possibly caused by prevent preferential amplification of the gfp target, an oligonucleotide limitation experiment for the gfp reaction was performed (section 2.13.3). By limiting the amount of gfp oligonucleotides, the target is amplified, but as the PCR progresses, the gfp oligonucleotides are depleted and the gfp reaction is limited. Limiting the gfp reaction should prevent gfp amplification competing with the target reaction and reduce the loss of sensitivity of the assay. Surface graphs were prepared to show the effect of each oligonucleotide concentration upon either the $C_T$ (Figure 7.1) or $R_n$ (Figure 7.2). Optimal conditions are shown in blue with sub-optimal in red. The lowest oligonucleotide combination consisted of 50nM and 200 nM concentrations per reaction for the forward and reverse gfp oligonucleotides respectively that produced an optimal $R_n$ value of 1.0 (indicating a sufficient increase in fluorescence for detection) without loss of sensitivity, $C_T = 29$ (Figure 7.2).

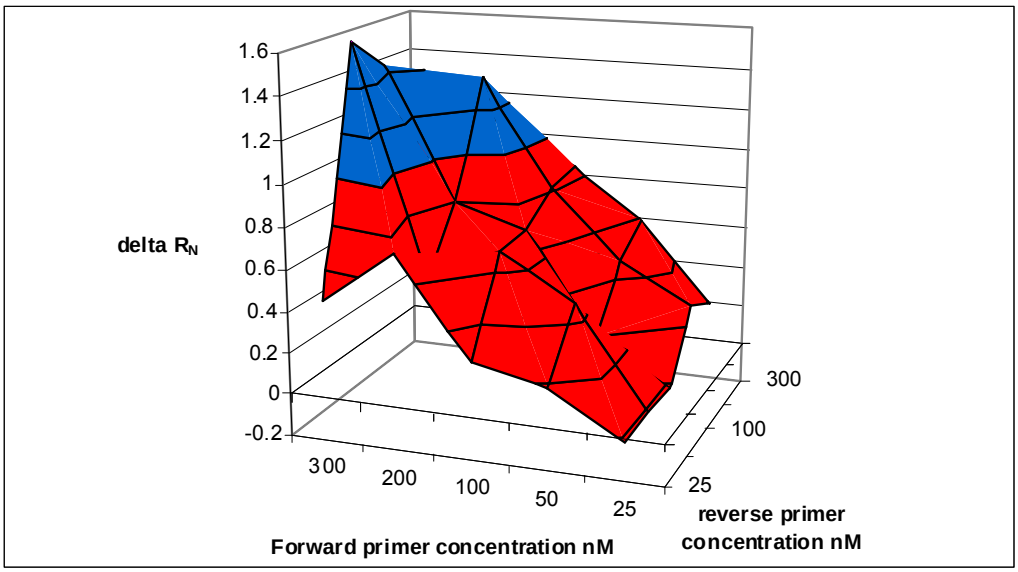



**Figure 7.1 Oligonucleotide matrix for gfp PCR showing the effect of the oligonucleotide concentrations on the delta Rn.**

Delta $R_n$ = normalized reporter signal; red = not acceptable delta $R_n$ value; blue = acceptable delta $R_n$ value.

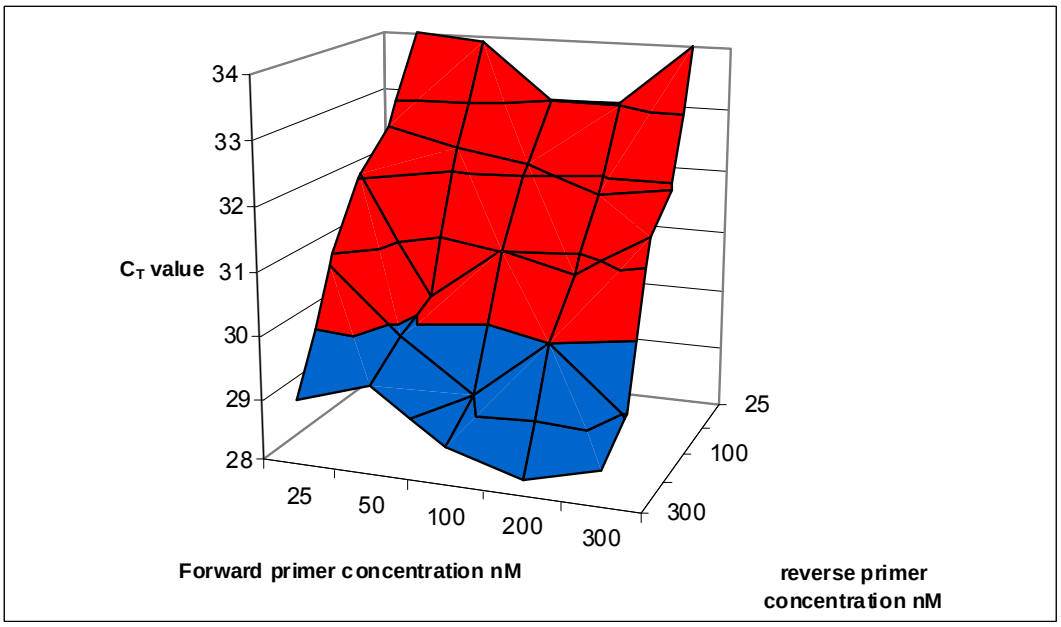



**Figure 7.2 Oligonucleotide matrix for gfp PCR showing the effect of the oligonucleotide concentrations on the $C_T$ value**.

$C_T$ = cycle threshold; red = not acceptable $C_T$ value; blue = acceptable $C_T$ value;

### 7.2.3 **Optimisation of the process control PCR assays: eaeγ, ceuE, mapA, hlyA and iroB reactions duplexed with gfp**

The optimised gfp reaction was duplexed with each of the pathogen target genes: *eae*γ, *ceuE, mapA, hlyA* and *iroB* and each duplex PCR assay was optimised by probe titration for the pathogen reaction (2.9.9). The lowest level of detection, $\Delta R_{n,}$ for each reaction in the duplex and the expected $C_T$ value for the gfp reaction in an optimised PCR reaction (i.e. no inhibition or loss of sensitivity) were established (Table 7.2). Analysis of the sensitivity of the PCR assays for the detection of *L. monocytogenes*, *C. coli, C. jejuni, E. coli* O157:H7 and *S. enterica* (sections 6.2.1-6.2.6 and 6.2.9) combined with the gfp reaction was performed in duplicate for each of the assays with concordant results obtained. An optimal $\Delta R_n$ value was considered as 1.0 or above, which indicates a sufficient increase in fluorescence for detection.

The *C. coli* PCR (*ceuE*: *gfp)* was optimised for the gfp target by increasing the gfp probe concentration from 100nM to 150 nM and decreasing the ceuE probe concentration from 100nm to 50 nM per reaction. The *L. monocytogenes* PCR (hlyA:gfp) assay was optimised by doubling the *hlyA* probe concentration to 200 nM per reaction. The *C. jejuni* PCR (mapA:gfp) was optimised by increasing the gfp probe concentration to 125 nM per reaction. The *E. coli* O157:H7 PCR (eaeγ:gfp) used standard gfp and eaeγ probe concentrations of 100 nM per reaction. The *S. enterica* PCR (iroB:gfp) was optimised by reducing the iroB probe concentration to 35 nM per reaction.

The optimised process control assays were applied to a serial dilution of an overnight culture of each target organism (Table 7.2). The lowest level of detection of the PCR assay was determined by plotting a standard curve of $C_T$ verses ACC. The lowest level of detection of *C. coli* was approximately 1.4 CFU per PCR. The lowest level of detection for *C. jejuni* was 70 CFU per PCR. The lowest level of detection of *E. coli* O157:H7 was approximately 210 CFU per PCR. The lowest level of detection of *L. monocytogenes* was 10 CFU per PCR. The lowest level of detection of *S. enterica* was 15 CFU per PCR.

**Table 7.2 Summary of performance of the re-optimised PCR assays when duplexed with gfp internal control and applied to enrichment cultures.**

| Target organism | Broths | Target gene | Lowest level of detection | | Target gene $\Delta R_n$ | *gfp* gene | |
|---|---|---|---|---|---|---|---|
| | | | CFU/ PCR | $CFUml^{-1}$ | | $\Delta R_n$ | $C_T$ |
| *C. coli* (NCTC 12110) | Bolton | *ceuE* | 1.4 | 28 | 1.5 | 0.7 | 30 |
| *C. jejuni* (NCTC 11168) | Bolton | *mapA* | 70 | 1400 | 1.5 | 1.2 | 28 |
| *E. coli* O157:H7 (NCTC 12900) | mTSB | *eaeγ* | 210 | 4200 | 1.4 | 1.1 | 28 |
| *L. monocytogenes* (NCTC 12427) | Half Fraser | *hlyA* | 10 | 200 | 1.2 | 1.1 | 28 |
| *S. enterica* (NCTC 13349) | BPW, RVS, SC, MKTTn | *iroB* | 15 | 300 | 1.5 | 1.3 | 28 |

$C_T$ = cycle threshold; CFU = colony forming unit. $\Delta R_n$ = normalized reporter signal; mTSB = modified Tryptone Soy Broth, BPW= Buffered Peptone Water, RVS = Rappaport Vassiliadis Soya Peptone Broth, SC = Selenite Cystine Broth, MKTTn = Muller Kauffman Tetrathionate Broth.

### 7.2.4 Application of optimised process control assays to un-inoculated broths to establish if broths were inhibitory to the PCR assays

Un-inoculated broths with and without the addition of a Lenticule disc containing $10^4$ *E. coli* GFP were tested in each duplex assay (section 2.9.9). Broths tested included: BPW, RVS, SC and MKTTn for *S. enterica* enrichment, mTSB for *E. coli* O157:H7, Bolton Broth for *C. coli* and *C. jejuni*, and half and full strength Fraser Broths for *L. monocytogenes* enrichment. After MagNA Pure™ DNA extraction, $C_T$ values were greater than 40 for the eaeγ, ceuE, mapA, hlyA and iroB reactions of the assays indicating there was no amplification of any of these gene fragments. No amplification for the *gfp*

gene was detected ($C_T$ > 40) in any of the broths in the absence of a Lenticule disc. Where an *E. coli* GFP Lenticule disc was included, $C_T$ values of 27 to 29 were detected, indicating no PCR inhibition resulted from the components of the broths and that the gfp reaction had performed optimally. The $C_T$ value obtained from the Lenticule in an un-inoculated broth was used as a reference for determining PCR inhibition or loss of sensitivity in the field trial.

Following the introduction of $10^4$ E. coli containing the *gfp* gene, as an PC, to one ml of enrichment broth, a mean $C_T$ value in the gfp component of the assays of <32.1 was taken to indicate successful DNA extraction without residual inhibition. PCR inhibition was recorded as a mean $C_T$ value of > 40 in the gfp reaction of the assay, and loss of sensitivity as a mean $C_T$ values of between ≥ 32.2 and ≤ 40, which is equivalent to a greater than one log reduction in sensitivity.

### 7.2.5 **Results: Application of PCR assays incorporating internal positive control to detect bacterial pathogens in naturally-contaminated food samples**

The real-time PCR assays with the internal process control (IPC) were first applied to 15 food homogenates i.e. food samples that had not been culturally enriched for the detection of *S. aureus* and *C. perfringens* (section 2.13.4). Unfortunately, the PCR was not sufficiently sensitive to detect foodborne pathogens without prior enrichment culture so all further assays were applied to enrichment cultures. The real-time PCR assays were then applied to a total of 42 enrichment cultures for the detection of *S. aureus, C. perfringens* or *L. monocytogenes*.

The PCR and culture methods for all the samples tested in this section are as flows. DNA was extracted from the food homogenates or enrichment cultures and the resulting DNA was amplified and detected by real-time PCR (section 2.13.4). All the food samples were also tested by the Food, Water and Environmental laboratory in London using culture detection techniques (section 2.13.1). Results of the PCR assays and culture applied to the enrichment broth cultures are described in the following sections.

### 7.2.6 **Direct PCR detection *S. aureus* and *C. perfringens* in food samples**

Direct PCR detection, without prior cultural enrichment, of *S. aureus* and *C. perfringens* was investigated as a rapid analysis method i.e. taking less than 24 hours, as no enrichment culture was required. The cpe IPC and nuc IPC assays were applied directly to food homogenates for the detection of *C. perfringens* enterotoxin gene and *S. aureus* thermonuclease gene and the results were compared to cultural analysis (Table 7.3).

**Table 7.3 Comparison of PCR assays and culture for the detection of *S. aureus* and *C. perfringens* in naturally-contaminated food homogenates without prior enrichment culture.**

| Assay | Number of samples where bacterial target detected | | Concordance |
|---|---|---|---|
| | PCR (inhibition) | [1]Culture | |
| *S. aureus nuc* | 0/9 (ND) | 0/9 | 100% |
| *C. perfringens cpe* | 0/6 (ND) | 0/6 | 100% |

ND = not detected, [1]Culture methods described in section 2.4.4.

Nine homogenised food samples (without prior enrichment culture) were analysed for the presence of the *nuc* gene and six homogenised food samples were analysed for *cpe* genes by PCR. The *nuc* or *cpe* gene targets were not detected by PCR ($C_T$ = 40) or culture analysis. No inhibition to the cpe PCR or the nuc PCR was detected when applied to DNA samples extracted from food samples prior to enrichment. The positive controls were detected in the PCR assays indicating valid runs.

These samples probably did not contain the target organisms, as they were not detected by culture. Although this approach was rapid, it was decided to test the remaining samples after cultural enrichment. Enrichment allows resuscitation of the bacterial cells, viable cells will outgrow DNA from dead cells and provides a greater number of target cells.

### 7.2.7 *C. perfringens* IPC PCR compared to culture analysis of enrichment cultures inoculated naturally-contaminated food and swab samples

Samples of canned foods were tested for the presence of *C. perfringens* as part of a survey for the London Port Health Authorities in to the microbiological safety of imported food products. For this survey, the food samples were enriched by culture in Cooked Meat Broth for the detection of sulphite reducing *C. perfringens*. The cpe IPC and cpa and IPC PCR assays were

applied to 14 Cooked Meat Broth enrichment cultures inoculated with five beef and nine meat (meat type unspecified) samples (Table 7.4).

**Table 7.4 Comparison of *C. perfringens* cpe and cpa IPC PCR to culture analysis of cooked meat enrichment cultures inoculated with samples of canned foods.**

| Sample type | PCR result ($C_T$) | | | | [1]Culture result |
|---|---|---|---|---|---|
| | Alphatoxin duplex | | Enterotoxin duplex | | |
| | *cpa* gene | IPC target | *cpe* gene | IPC target | |
| Canned beef | Detected (34) | Detected (23) | ND (40) | Detected (24) | ND |
| | Detected (33) | Detected (23) | ND | Detected (24) | ND |
| | Detected (33) | Detected (24) | ND | Detected (24) | ND |
| | Detected (32) | Detected (24) | ND | Detected (24) | ND |
| | Detected (29) | Detected (24) | ND | Detected (24) | ND |
| Canned meat (meat type unspecified) | Detected (34) | Detected (25) | ND | Detected (24) | ND |
| | Detected (36) | Detected (24) | ND | Detected (23) | ND |
| | Detected (36) | Detected (31) | ND | Detected (24) | ND |
| | Detected (35) | Detected (26) | ND | Detected (23) | ND |
| | Detected (35) | Detected (24) | ND | Detected (24) | ND |
| | Detected (34) | Detected (24) | ND | Detected (24) | ND |
| | Detected (33) | Detected (24) | ND | Detected (24) | ND |
| | Detected (33) | Detected (24) | ND | Detected (24) | ND |
| | ND (40) | Detected (23) | ND | Detected (24) | ND |
| Positive control | Detected (18) | Detected (27) | Detected (35) | ND (40) | n/t |
| NTC | ND (40) | Detected (24) | ND (40) | Detected (24) | n/t |
| NAC | ND (40) | ND (40) | ND (40) | ND (40) | n/t |
| Un-inoculated CMB | Detected (35) | Detected (23) | ND (40) | Detected (24) | ND |

[1]Culture methods described in section 2.4.4. $C_T$ = cycle threshold**;** IPC = internal positive control; ND = not detected; NTC = non-template control; NAC non-amplification control; N/t = not tested; CMB =Cooked Meat Broth.

The *C. perfringens cpa* gene fragment was detected in 13 of 14 samples, however the *cpe* gene fragment or *C. perfringens* isolates were not identified any of the 14 samples. PCR inhibition was not detected in any of the samples suggesting that the negative cpe result were not due to PCR inhibition. The *cpa* gene fragment detection may have been due to cross-contamination although

*cpa* gene fragment was not detected in the DNA extraction or PCR negative controls. A sample of un-inoculated Cooked Meat Broth was subjected to MagNa Pure™ DNA extraction and PCR analysis for *cpe* and *cpa* gene fragments. The result were identical to the sample analysis with *cpa* gene fragment detected ($C_T$ = 35), *cpe* gene fragment not detected ($C_T$ = 40) and *C. perfringens* not isolated. This result indicated that the anomaly was caused by the Cooked Meat Broth.

### 7.2.8 *S. aureus* IPC PCR compared to culture analysis of enrichment cultures inoculated naturally-contaminated food and swab samples

The nuc IPC duplex was applied to two sets of samples tested from two food poisoning outbreaks. The cultural analysis was applied to the food homogenates prior to enrichment, however, the PCR assay was applied to BPW (Buffered Peptone Water) enrichment cultures that had been inoculated with the 12 food samples (Table 7.5).

**Table 7.5 IPC nuc PCR for the detection of *S. aureus* in homogenised food samples compared with cultural analysis**.

| Food poisoning | Sample type | PCR target ($C_T$) | | [1]Culture $CFUg^{-1}$ |
|---|---|---|---|---|
| | | *nuc* gene | IPC | |
| Outbreak 1 | Dosa (pancakes, lentils and carrot) | **Detected (39)** | Detected (24) | **40** |
| | Kothu Rotti (meat and rotti) | **Detected (18)** | Detected (25) | **3500** |
| | Vegetarian curry mix | ND (40) | Detected (24) | **20** |
| | Idli (rice cakes, lentils and carrot) | ND (40) | Detected (24) | **20** |
| | Rice | ND(40) | Detected (24) | ND |
| Outbreak 2 | Meat and bone | **Detected (38)** | Detected (26) | ND |
| | Meat and stuffing | **Detected (35)** | Detected (26) | ND |
| | Meat and vegetables | **Detected (38)** | Detected (26) | ND |
| | Pork meat | **Detected (36)** | Detected (26) | ND |
| | Bolognese sauce | ND (40) | Detected (26) | ND |
| | Meat pate | ND(40) | Detected (26) | ND |
| | Undefined | ND(40) | Detected (26) | ND |
| Positive control | | Detected (22) | Detected (27) | n/t |
| Negative control | | ND (40) | Detected (24) | n/t |
| Non-amplification control | | ND (40) | ND (40) | n/t |

[1]Culture methods described in section 2.4.4. $C_T$ = cycle threshold; IPC = internal positive control; ND = not detected, n/t =not tested. **Samples positive for the target are shown in bold**.

Six food samples were PCR positive for the *S. aureus* thermonuclease (*nuc*) gene. One sample of Kothu Rotti (Sri Lankan meat and bread ready-to-eat meal with spices) was found to be positive for the thermonuclease (*nuc*) gene ($C_T$ = 18), which was confirmed by cultural analysis. The *S. aureus* thermonuclease (*nuc*) gene was detected ($C_T$ = 39) in a sample of Dosa (rice cakes, lentils and carrot) and confirmed by cultural analysis. *S. aureus* thermonuclease (*nuc*) gene was detected in a further four foods (meat and bone; meat and stuffing; meat and vegetables; and pork meat), however *S. aureus* was not detected by culture. This result may be a false positive PCR result or a false negative culture result and should have been repeated, but the samples had unfortunately been discarded.

In two samples of vegetarian curry mix and Idli (rice cakes, lentils and carrot), the PCR failed to detect the *S. aureus* thermonuclease (*nuc*) gene, but *S. aureus* was detected by culture (20 $CFUg^{-1}$). The false negative PCR result for these two samples was not due to PCR inhibition because the IPC was detected, so may have been due to the low level of cells in the samples. In comparison, the Dosa (pancakes, lentils and carrot) sample (containing 40 $CFUg^{-1}$ of *S. aureus*) was considered positive with a $C_T$ of 39, which is at the limit of detection for this assay.

### 7.2.9 *L. monocytogenes* IPC PCR compared to culture analysis of enrichment cultures inoculated naturally-contaminated food and swab samples

Sixteen Half Fraser primary enrichment cultures inoculated with nine sandwich and seven environmental swab samples were received during a *Listeria* contamination incident at a sandwich producing factory. The samples were tested for *Listeria* spp. by cultural analysis and *L. monocytogenes* using *hlyA* IPC PCR (Table 7.6). *L. monocytogenes* was detected in nine (56%) samples, consisting of three environmental swabs and six sandwiches. All nine samples where the *L. monocytogenes hlyA* gene was detected were culture

confirmed positive for *L. monocytogenes* indicating a 100% concordance between the PCR and culture results.

**Table 7.6 IPC hlyA PCR results for the detection of *L. monocytogenes* in enrichment cultures inoculated with food and environmental samples associated with a *Listeria* contamination incident at a sandwich producing factory**.

| Sample type | PCR result ($C_T$) | | [1]*L. monocytogenes* |
|---|---|---|---|
| | *hlyA* gene | IPC | culture |
| Environmental | **Detected (35)** | Detected (30) | **Detected** |
| swabs | **Detected (23)** | Detected (31) | **Detected** |
| | **Detected (22)** | Detected (31) | **Detected** |
| | ND (40) | Detected (29) | ND |
| | ND (40) | Detected (29) | ND |
| | ND (40) | Detected (30) | ND |
| Sandwiches | **Detected (37)** | Detected (29) | **Detected** |
| | **Detected (35)** | Detected (30) | **Detected** |
| | **Detected (34)** | Detected (29) | **Detected** |
| | **Detected (24)** | Detected (30) | **Detected** |
| | **Detected (24)** | Detected (30) | **Detected** |
| | **Detected (22)** | Detected (31) | **Detected** |
| | ND (40) | Detected (29) | ND |
| | ND (40) | Detected (29) | ND |
| | ND (40) | Detected (29) | ND |
| Positive control | Detected (17) | Detected (39) | n/t |
| Negative control | ND (40) | Detected (30) | n/t |
| NAC | ND (40) | ND (40) | n/t |

[1]Culture methods described in section 2.4.4. $C_T$ = cycle threshold; IPC = internal positive control, NAC non-amplification control; n/t = not tested. ND = not determined. **Samples positive for the target are shown in bold**.

## 7.3 Results: Application of process control PCR assays to enrichment cultures inoculated with naturally-contaminated food samples in a field trial.

The real-time PCR assays with the process control (PC) were applied to 357 enrichment cultures that had been inoculated with naturally-contaminated food samples. Broth enrichment cultures were inoculated with food samples and the resulting primary or secondary enrichment cultures were tested by PCR. The PCR and culture methods for all the samples tested in section 7.3 and 7.4 are as flows. Aliquots of the cultures (1ml) were spiked with a Lenticule and DNA was extracted, the resulting DNA was amplified and detected by real-time PCR (section 2.13.5). All the food samples were also tested by the Food, Water and Environmental laboratories in Preston and London using culture detection techniques (section 2.13.1). Results of the PCR assays and culture applied to the enrichment broth cultures are summarised in Table 7.7 and described in the following sections.

### 7.3.1 *Campylobacter* spp. PCR compared to culture analysis of enrichment cultures inoculated with naturally-contaminated food samples

Fifty three Bolton Broth enrichment cultures were inoculated with 52 food samples (one sample was tested in duplicate) for the cultural detection of *Campylobacter* spp. Four food enrichment cultures were PCR positive for *C. jejuni,* two of which were also *C. coli* PCR positive. The remaining two *C. jejuni* PCR positive samples were from cooked chicken and egg mayonnaise enrichment samples, but neither of these samples was confirmed by culture methods as positive for *Campylobacter* spp. No inhibition was detected in either PCR assay on DNA extracted from Bolton Broth enrichment culture. However, a loss of sensitivity was detected in two enrichment cultures (one inoculated with barbeque chicken and one with raw crab) using the ceuE:gfp assay for the detection of *C. coli*, and one (inoculated with a chicken sample) using the mapA:gfp *for* the detection of *C. jejuni* PCR.

### 7.3.2 *E. coli* O157:H7 PCR compared to culture analysis of enrichment cultures inoculated with naturally-contaminated food samples

Five modified Tryptone Soy Broth (mTSB) enrichment cultures were inoculated with goat's cheese samples for the detection of *E. coli* O157:H7. None of these samples was found to be positive either by the *eae*γ gene PCR or by culture for *E. coli* O157:H7. No inhibition or loss of sensitivity to the PCR assay was observed using DNA extracted directly from the mTSB enrichment Broths (as indicated by the PC).

### 7.3.3 *L. monocytogenes* PCR compared to culture analysis of enrichment cultures inoculated with naturally-contaminated food samples

A total of 126 enrichment broths were analysed for the detection of *Listeria* spp. using the hlyA:gfp PCR assay. Of the 126 broths, 83 were half strength Fraser Broths used for primary enrichment and 43 were Fraser Broths for secondary enrichment. The broths were inoculated with 107 food samples, and replicate Fraser Broths were prepared for six food samples. The *hlyA* gene fragment was detected in six enrichment cultures (five from primary enrichment and one in a secondary enrichment broth) and the presence of *L. monocytogenes* was confirmed by cultural analysis in all these samples (Table 7.7). The *hlyA* gene fragment was detected in an additional seven primary enrichment broths (half Fraser) where *L. monocytogenes* was not detected by conventional culture methods.

Concordant *L. monocytogenes hlyA* PCR positive results were obtained from replicate Fraser Broths prepared from 12 samples. The samples were six food (five cooked meat samples and one ice cream), five environmental swabs and two unidentified samples. PCR inhibition was detected in one enrichment culture inoculated with chocolate fudge cake: this sample was *L. monocytogenes* negative by both *hlyA* gene PCR and by culture. A loss of assay sensitivity was detected in one enrichment culture inoculated with a cheese sample, which was negative for both *hlyA* gene and *L. monocytogenes*

by culture. The high fat content of chocolate fudge cake and cheese may have caused inhibition to the internal control reaction causing the loss of assay sensitivity.

### 7.3.4 *S. enterica* PCR compared to culture analysis of enrichment cultures inoculated with naturally-contaminated food samples

Enrichment broths were inoculated with 120 food samples for the detection of *Salmonella* spp. *S. enterica* was isolated from one of the primary and secondary enrichment broths and the *iroB* gene fragment was detected by PCR. This positive sample consisted of a Dosa (rice cakes with lentils and carrots), and as well as *S. enterica*, this sample was also positive for *S. aureus* (section 7.2.4), which indicated that the food had been poorly handled. PCR inhibition of the iroB:gfp was detected in eight enrichment cultures inoculated with herb samples, and a loss of PCR sensitivity was detected in BPW *S. enterica* enrichment cultures inoculated with two herbs and one yoghurt sample. Using DNA extracted from the enrichment broths and detection of the *gfp* gene as an PC, no inhibition or loss of sensitivity was detected in 107 of the samples, inhibition or loss of sensitivity was detected in 12 of 120 (10%) samples (Table 7.7). *Salmonella* spp. was not isolated by culture from any of the broths where PCR was inhibited or where the PC detected loss of sensitivity.

**Table 7.7 PCR assays applied to the field trial enrichment broth cultures from food and environmental enrichment broth cultures.**

| Culture type | Enrichment broth | Total numbers | Number of broths | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | [1]Culture result | | PCR result | | Internal control (*gfp* gene detection) | |
| | | | Detected | Not detected | Detected | Not detected | Loss of sensitivity | Inhibition |
| *Campylobacter jejuni* | | | | | | | | |
| Primary enrichment | BB | 53 | 0 | 53 | 4 | 49 | 1 | 0 |
| *Campylobacter coli* | | | | | | | | |
| Primary enrichment | BB | 53 | 0 | 53 | 2 | 51 | 2 | 0 |
| *Escherichia coli* O157:H7 | | | | | | | | |
| Primary enrichment | mTSB | 5 | 0 | 5 | 0 | 5 | 0 | 0 |
| *Listeria monocytogenes* | | | | | | | | |
| Primary enrichment | HF | 83 | 5 | 78 | 12 | 71 | 1 | 1 |
| Secondary enrichment | FB | 43 | 1 | 42 | 1 | 42 | 0 | 0 |
| *Salmonella enterica* | | | | | | | | |
| Primary enrichment | BPW | 120 | 1 | 119 | 1 | 119 | 4 | 8 |
| Totals: | | 357 | 7 | 350 | 20 | 337 | 9 | 10 |

[1]Culture methods described in section 2.4.4. BB = Bolton Broth; mTSB = modified Tryptone Soy Broth, FB = Fraser Broth, HF = Half Fraser Broth; BPW= Buffered Peptone Water; gfp $C_T$ < 32.1 = no inhibition; gfp $C_T$ 32.1- 40 = loss of sensitivity; gfp $C_T$ > 40 = inhibition.

## 7.4 Results: Application of the *S. enterica* real time PCR incorporating the process control in an investigation of contamination of raw shell eggs and food poisoning incident.

Samples from two incidences associated with *Salmonella* were analysed as part of the work performed as part of this study. The first incident was an investigation into potential *Salmonella* contamination of raw shell egg samples performed by the London Food, Water and Environmental Laboratory as on-going surveillance. The second incident was part of an investigation of an outbreak of *S. enterica* serovar Agbeni in a care home performed by the Preston Food, Water and Environmental Laboratory. The results of the PCR and culture analysis are as follows.

### 7.4.1 Detection of *S. enterica* in egg samples using the iroB:gfp PCR

Egg samples were grouped into batches of six eggs and the shells separated from the contents and inoculated into 117 BPW enrichment cultures (58 eggshell and 59 egg content samples) by the London F.W.E. laboratory. The *iroB* gene specific for *S. enterica* was detected in 18 of 117 (15%) egg enrichment samples, of these 14 of 117 (12%) were confirmed by culture as containing *S. enterica*. The 18 PCR positive samples for the *iroB* gene of *S. enterica* consisted of two egg contents and 16 eggshell enrichment cultures. *S. enterica* was isolated from one additional shell sample; however, the *iroB* gene was not detected by PCR. None of the BPW enrichment cultures inoculated with egg samples inhibited the iroB:gfp real-time PCR (defined by the PC gfp amplification giving Ct values of between 26 and 28) however, a loss of sensitivity ($C_T$ 30 - 40) was detected for 13 samples consisting of 12 enrichment cultures of egg contents and one culture from an eggshell.

Serotyping and phagetyping were performed by the Salmonella Reference Laboratory (HPA) on one isolate from each of the 14 culture confirmed samples supplied by the London F.W.E. laboratory. Twelve of the egg samples contained *S. enterica* serovar Enteritidis phage type one; one type seven and

one contained an untyped *Salmonella* spp. One *S. enterica* serovar Enteritidis phage type four was isolated from an egg sample, but was negative for the *iroB* gene fragment in BPW primary enrichment culture. The egg samples comprised of four from England, 34 from France, four from Ireland and 75 from Spain. The *iroB* gene fragment was detected in 18 eggs samples, 17 of which originated from Spain and one from France. The egg samples from Spain were all culture confirmed as containing *S. enterica* serovar Enteritidis. The iroB PCR positive sample from France was not culture confirmed as positive for *S. enterica*. The results are summarised in Table 7.8.

**Table 7.8 PCR detection of *S. enterica iroB* gene in egg samples.**

| Sample type (number of samples) | S. *enterica* detected | | Phage type (number of isolates) | Country of origin for positives |
|---|---|---|---|---|
| | PCR (*iroB* gene detected) | [1]Culture confirmed | | |
| Eggshell (58) | 16 | 13 | Type 1 (12)<br>Type 7 (1) | Spain |
| Egg content (59) | 2 | 1 | [2]Type 4 (1) | Spain (Culture confirmed)<br>France (PCR positive) |
| Total: 117 | 18 | 14 | 14 | |

[1]Culture methods described in section 2.4.4. [2]includes one sample not detected by the PCR.

### 7.4.2 Application of the *S. enterica* PCR to samples from a laboratory confirmed outbreak of *S. enterica* serovar Agbeni in a care home.

A laboratory confirmed outbreak of *S. enterica* serovar Agbeni in a care home in South Manchester resulted in six people being taken ill in June 2005. From the microbiological investigation, 29 enrichment cultures were provided by Preston FWE laboratory for PCR testing in this study and results shown in Table 7.9. The samples consisted of environmental swab and vacuum cleaner dust samples that were first cultured in BPW. The BPW enrichment cultures were then selectively enriched in Rappaport Vassiliadis Soya Peptone Broth (RVS) and Selenite Cystine Broth (SC), apart from two samples that where

Muller Kauffman Tetrathionate Broth with novobiocin (MKTTn) replaced either SC or RVS.

*S. enterica* serovar Agbeni was detected in each sample by culture. A positive result was recorded by the culture method if *Salmonella* spp. were cultured from one or both of the selective enrichment cultures after further culture on solid selective media following confirmation using biochemical tests. The PCR was applied to all broths provided, but not to isolates from the cultural detection. *S. enterica iroB* gene was detected in all the samples and 27/29 broth cultures. *S. enterica iroB* gene was not detected in the SC enrichment culture for sample 4 and the BPW culture for sample 11 (Table 7.9).

**Table 7.9 PCR detection of *S. enterica iroB* gene from primary and secondary enrichment broths from confirmed outbreak of *S. enterica* serovar Agbeni.**

| Sample number | S. *enterica* enrichment culture ($C_T$) | | | | [1]Final Salmonella culture result |
|---|---|---|---|---|---|
| | Primary enrichment culture (BPW) | Secondary enrichment culture | | | |
| | | SC | RVS | MKTTn | |
| 1 | n/t | Detected (29) | Detected (19) | n/t | Detected |
| 2 | n/t | Detected (32) | Detected (18) | n/t | Detected |
| 3 | n/t | Detected (25) | Detected (18) | n/t | Detected |
| 4 | n/t | **ND (40)** | Detected (22) | n/t | Detected |
| 5 | n/t | Detected (31) | Detected (18) | n/t | Detected |
| 6 | n/t | Detected (29) | Detected (20) | n/t | Detected |
| 7 | Detected (18) | Detected (18) | Detected (18) | n/t | Detected |
| 8 | Detected (17) | Detected (22) | Detected (17) | n/t | Detected |
| 9 | Detected (18) | Detected (19) | Detected (17) | n/t | Detected |
| 10 | Detected (21) | Detected (28) | Detected (18) | n/t | Detected |
| 11 | **ND (40)** | Detected (25) | n/t | Detected (27) | Detected |
| 12 | n/t | n/t | Detected (18) | Detected (20) | Detected |
| PCR positive control | | | Detected (29) | | n/t |
| PCR negative control | | | ND (40) | | n/t |

[1]Culture methods described in section 2.4.4.BPW= Buffered Peptone Water, RVS = Rappaport Vassiliadis Soya Peptone Broth, Selenite = Selenite Cystine Broth, MKTTn = Muller Kauffman Tetrathionate Broth. $C_T$ = cycle threshold. BPW was the primary enrichment medium with the other broths used for selective enrichment. n/t = not tested. ND = not detected.

The *S. enterica iroB* gene may not have been detected in samples 4 and 11 due to low levels of *Salmonella* in the sample, however for sample 11, *S. enterica iroB* gene was detected following a further enrichment in selective broth culture. Sample 4 was not detected by PCR after enrichment in SC but was following enrichment in RVS. The $C_T$ values were generally lower from amplification of the *iroB* gene in the RVS enrichment cultures compared to the SC cultures (Table 7.8). This may be due to more *iroB* gene target in the RVS cultures or the assay being more sensitive using the RVS Broth. However, the process control resulted in a mean $C_T$ 28.4 from SC cultures and $C_T$ 29.1 from RVS cultures and this would suggest that the assay was more sensitive when applied to cultures enriched with SC. Possibly, this was because there was less *iroB* gene target competing for PCR reagents. Unfortunately, as only two MKTTn cultures were tested it was not possible to compare MKTTn with either SC or RVS accurately. All the samples were tested with the process control that used the gfp reaction. $C_T$ values = $\leq$32.2 indicated that there was no loss of PCR sensitivity or inhibition.

### 7.5 Summary of findings from the IPC and PC assays

A summary of all the finding for analysis of the food samples is presented in Table 7.10. In 62 of 558 samples tested a target gene was detected by PCR. Of these, 36 were culture confirmed as containing the bacterial pathogen associated with the PCR result. Ideally, the samples should have been re-tested, however, food samples must be tested within 24 hours or kept frozen to give a representative indication of the levels of contamination.

PCR inhibition or loss of sensitivity was detected in 31 of the 558 samples. Inhibition was detected in 10 samples, a chocolate fudge cake enrichment for the detection of *L. monocytogenes,* in eight herb samples enrichments for the detection of *Salmonella* and one canned food screened for the presence of *C. perfringens*. A loss of PCR sensitivity was detected in 21 samples, three in the ceuE assay for the detection of *C. coli,* one in the hlyA assay for *L. monocytogenes,* 17 in the iroB for the detection of *S. enterica,* one in the

mapA for the detection of *C. jejuni* and one cpa for the detection of *C. perfringens*. The loss of sensitivity in the PCR assays occurred in chicken, crab, cheese, egg contents, eggshell, herbs, pickle, yoghurt and canned food enrichment samples.

**Table 7.10 Overview of field trial results.**

| Test | Target bacteria (PCR assay) *C. coli* (ceuE) | *L .monocytogenes* (hlyA) | | *S. enterica* (iroB) | *C. jejuni* (mapA) | *C. perfringens* (cpa) | *S. aureus* (nuc) | *E. coli* O157:H7 (eaey) | Total |
|---|---|---|---|---|---|---|---|---|---|
| Internal control type | PC only | IPC | PC | PC only | PC only | IPC only | IPC only | PC only | |
| Total number of broths tested | 52 | 126 | 16 | 266 | 52 | 20 | 21 | 5 | 558 |
| Food sample type in which organisms was PCR | Raw chicken (2) | Cooked meat (1), cooked turkey (2), cooked beef (1), ice-cream (1), Ham (1), Swabs (5) | Swabs (3) sandwiches (6) | Swabs (13), Eggs (17) Dosa (1) | Raw chicken (2), mayonnaise (1), Cooked chicken (1). | ND | Kothu Rotti, Dosa, Pork meat, meat and bone, meat and vegetables, meat and stuffing (6) | ND | 62 |
| Organism detected by culture | ND | Cooked meat (1), cooked turkey (2), cooked beef (1), ice-cream (1) | culture confirmed | Swabs (13), Eggs (15) Dosa (1) | mayonnaise (1), Cooked chicken (1) | ND | Culture confirmed | ND | 36 |
| Inhibition | ND | ND | Chocolate fudge cake (1) | Herbs (8) | ND | Canned food (1) | ND | ND | 10 |
| Loss of sensitivity | Crab (1), Chicken (2) | ND | ND | Egg content (12) Egg shell (1) Herbs (2) Pickle (1) Yoghurt (1) | Chicken (1) | ND | ND | ND | 21 |
| Organism detected by PCR or [1]culture in inhibited samples | ND | ND | ND | ND | *C. jejuni* | ND | ND | ND | 1 |

[1]Culture methods described in section 2.4.4. $C_T$ = cycle threshold gfp $C_T$ < 32.1 = no inhibition; gfp $C_T$ 32.1- 40 = loss of sensitivity; gfp $C_T$ > 40 = inhibition; ND not detected; total number of positive samples shown in parenthesis.

## 7.6 Discussion

The aim of the work reported in this chapter was to optimise then apply the PCR assays that incorporated the MagNA Pure™ DNA extraction, the lenticulated *E. coli* GFP internal control or IPC for the detection of bacterial pathogens in naturally-contaminated food samples.

During the development of any new assay, a field trial of the technique is required as part of its validation prior to its implementation in a diagnostic laboratory testing schedule. In a field trial, a new or modified assay is applied to naturally-contaminated or clinical samples (as opposed to spiked samples) and where possible compared to equivalent results obtained from the current standard culture detection method.

### 7.6.1 **Optimisation of internal positive control PCR assays and internal process control assays for the detection of bacterial pathogens in naturally-contaminated food samples**

Real-time PCR assays for the detection of *S. aureus nuc* gene, *L. monocytogenes hlyA* gene, and *C. perfringens cpe* and *cpa* genes were duplexed with a commercial IPC kit. No loss of sensitivity for either the target gene or the IPC DNA fragment was detected after optimisation of the duplex assays. The IPC monitors for false negative PCR results and can be used in any standard 5' nuclease real-time PCR assay.

The *E. coli* GFP internal positive control, provided as a Lenticule disc was added to each test reaction and detected using a gfp real-time PCR assay. This enabled the internal positive control of the assay and incorporated control of the DNA extraction.

The *gfp* PCR was duplexed with each pathogen-specific PCR assay. Initially, a loss of sensitivity was observed for the pathogens and this is most likely to

have been due to preferential amplification of the *gfp* gene fragment 'out-competing' the pathogen-specific amplification (which may only be present at low levels). However, this loss of sensitivity was overcome by a minor modification of the gfp reaction to limit the gfp-oligonucleotide concentrations. After re-optimisation, the original sensitivities for detection of *C. coli, L. monocytogenes* and *S. enterica* were then regained. A one-log CFU reduction in the lowest level of detection for *E. coli* O157:H7 and *C. jejuni* targets were found using the duplex assays. However, this was considered an acceptable increase since the sensitivities were still acceptable (210 and 70 CFU per PCR respectively). The duplexed assays were then used with the lenticulated *E. coli* GFP as a process control assay.

The PC assays function by adding a quantified heterogeneous target PC to a food enrichment sample prior to DNA extraction, whereby the PC and pathogen can be simultaneously amplified and detected in a PCR assay and any reduction in the PC amplification would suggest the presence of PCR inhibition (Rosenstraus *et al.,* 1998). It was demonstrated in the work of this thesis that an PC can be easily added to a sample prior to DNA extraction, and in an optimally functioning duplex assay, the *gfp* gene was amplified with a $C_T$ value of 29.

### 7.6.2 **Application of PCR with IPC for the detection of pathogenic bacteria in naturally-contaminated food samples**

The nuc PCR was applied to 12 food samples, and the *nuc* gene fragment was detected in six of these samples. Culture results were consistent for two samples consisting of Dosa and Kothu Rotti investigated as part of a food poisoning outbreak. The Dosa and Kothu Rotti contained 40 and 3500 $CFUg^{-1}$ respectively. PCR failed to detect *S. aureus nuc* gene fragment in two samples (vegetarian curry mix and Idli), however these samples contained low levels of (20 $CFUg^{-1}$) *S. aureus* in the samples. This may have been due to insufficient lysis of the cells during the DNA extraction, or a lack of sensitivity for the nuc PCR. The sensitivity of the nuc PCR was calculated as 10 genome copies per

PCR (section 6.2.4), which does not suggest a PCR sensitivity problem. The sensitivity of the PCR may have been reduced when it was applied to a food sample rather than to purified DNA. When the sensitivity of the nuc PCR was tested with as whole assay including DNA extraction using MagNa Pure™ the sensitivity was reduced to 7.8 x $10^3$ CFU/PCR suggesting DNA extraction may be problematic. As discussed in section 3.3 the DNA may be degraded by staphylococcal nucleases in the DNA preparation, which is causing the loss of sensitivity in the assay. If this hypothesis can be proved the DNA extraction method should be altered (recommendations given in section 3.3).

The PCR also detected the *nuc* gene fragment in four samples (meat and bone, meat and stuffing, meat and vegetables and in pork meat) where *S. aureus* was not detected by culture. This may have been non-specific amplification however, no cross-reactions were detected during the validation.

For detection of *L. monocytogenes,* the *hlyA* assay was applied to food enrichment cultures. The presence of *Listeria* in sandwiches, soft ripened cheeses, pâtés and vacuum or modified atmosphere packed cooked meat with a long shelf life causes the food to be potentially hazardous to health due to the organism's ability to multiply to significant levels during refrigerated storage (Kathariou, 2002). EC Microbiological Criteria specifications for some RTE foods (described in Table 1.3) and dairy products stipulate the absence of *L. monocytogenes* in 25 g or one g of food sample respectively, following enrichment culture in Fraser Broth (EC, 2007).

The 15 samples analysed in this work, including nine *hlyA* PCR positives were part of an investigation into an outbreak of *L. monocytogenes* associated with sandwiches produced by a catering company. Swabs of production faculties were obtained in addition to food samples because *Listeria* spp. are ubiquitous in the environment and can indicate substandard environmental cleanliness standards. In this outbreak, *L. monocytogenes, L. welshimeri* and *L. innocua* were detected within the manufacturing facility and in food samples. The *hlyA* PCR assay described in this thesis provided a confirmed presence or absence

result for *L. monocytogenes* within four hours from enrichment culture, in contrast to several days, which is a considerable time saving compared to current microbiological identification and confirmation approach. Rodriguez-Lazaro *et al.* (2005) applied their assay directly to enrichment cultures from a range of food samples and found that Fraser and Half Fraser broth, raw pork and raw or cold-smoked salmon were strongly inhibitory to PCR. However using the assay developed here little inhibition was observed and good correlation between the PCR and culture methods was obtained.

### 7.6.3 **Application of internal process control (PC) PCR assays for the detection of pathogens in food samples**

Standard cultural techniques for the detection of foodborne pathogens were compared to real-time PCR assay with process control for naturally-contaminated food samples. A positive disc control i.e. a sample consisting of one ml water and one disc was used to compare process control PCR assays the results from samples to monitor small changes from run to run or degradation of discs. The samples consisted of 503 enrichment broths that had been inoculated with 424 food samples and 15 environmental samples that were collected between July and August 2006. The samples tested consisted of three sets: 117 enrichment broths inoculated with egg samples as part of a survey; 29 enrichment broths prepared as part of a *Salmonella* outbreak and the remaining broth cultures were collected from on-going surveillance work. The application of the PCR assay for the detection of the target pathogens in these enrichment cultures is described in the following sections. The PC assay (containing *gfp*) was used to monitor for loss of sensitivity or inhibition in these PCR assays.

Inhibition and a loss of sensitivity were detected only at a very low level in these enrichment broths: in nine (2%) and 21 (4%) of the samples respectively. All of the observed PCR inhibition was observed in DNA extracted from primary (as opposed to secondary) enrichment culture samples. These primary enrichment cultures contained a greater proportion of the original sample food

matrix and therefore a higher concentration of any inhibitors associated with it, therefore dilution in the secondary enrichment broths reduces these problems.

Application of the duplex assays to samples from potentially naturally-contaminated food samples for the detection of *S. enterica, L. monocytogenes, C. jejuni, C. coli and E. coli* O157:H7 showed a high level of concordance between PCR and microbiological culture results. Overall, there was a very low level of contaminated samples detected by either method. Based on the findings of this work, PCR would appear to be a suitable alternative for rapid screening of food enrichment samples, since there was a high level of agreement in both conventional microbiology and PCR. Negative samples do not require further analysis and their elimination at an early stage would greatly reduce both the cost and time taken for pathogen detection and microbiological surveillance.

There were a small number of samples where the *iroB*, *hlyA, mapA* or *ceuE* gene fragments were detected by PCR, but which were not confirmed by culture. It is possible that these results could be due to technical laboratory error, non-specific amplification or cross-contamination with target nucleic acid. However, duplicate test results were always concordant, PCR assays were demonstrated to be 100% specific for their DNA targets when tested with DNA from a range of other bacterial species. Additionally the positive and negative controls results suggested that there was no laboratory error or cross-contamination. This indicates that the most likely explanations to account for the PCR positive/culture negative results are due to a higher sensitivity of the PCR methods or a result of the detection of target DNA from dead or sub-lethally injured organisms that were not recovered by conventional culture.

Josefsen *et al.* (2004) developed a quantitative PCR for the detection of *C. jejuni*, *C. coli* and *C. lari* incorporating an IPC and applied it to both spiked and naturally-contaminated chicken rinse samples. Chelex resin was used for DNA preparation and no PCR inhibition was detected from culture negative samples, however the authors report a loss of *Campylobacte*r DNA during the

extraction suggesting that an alternative method such as MagNA Pure™ extraction should be adopted.

Enrichment broth prepared from herbs and egg containing food groups resulted in the greatest number of reactions with PCR inhibition or loss of sensitivity. All of the PCR assays used in this study suffered from a lack of sensitivity or inhibition with at least some of the sample types tested and it was concluded that other than these two sample types the problem was not particular to any one assay or food types. It was not predictable when inhibition or a loss of sensitivity may occur, not least due to the highly diverse nature of some food sources, indicating the importance of a control strategy.

### 7.6.4 **Application of the *S. enterica* real time PCR incorporating the process control in an investigation of contamination of raw shell eggs and food poisoning incident.**

As part of this study, 120 egg specimens were tested for *S. enterica*. The *iroB* gene fragment was detected 15 % of the egg enrichment cultures (28 % of which were from eggshell and 3% from egg content enrichments), with 12 % of these being culture confirmed as positive for *S. enterica*. The culture confirmed samples were all serotyped as *S. enterica* Enteritidis with isolates from 12 samples being phage type one, one phage type seven and one untyped. Of the PCR positive samples, 95% of the egg samples originated from Spain, with a single example, from France. Prior to the chicken flock vaccination program in the UK, the *S. enterica* Enteritidis serovar was associated with both chickens and eggs in this country, but now is more associated with eggs used in catering premises that have been sourced from mainland Europe (Little *et al.,* 2007).

The egg samples were tested by PCR at the non-selective resuscitation enrichment stage (BPW primary enrichment) of the cultural analysis, whereas the culture results were assigned after further selective enrichment (secondary

enrichment) and sub-culturing on selective agars. The additional enrichment procedures enable a lower level of detection than primary enrichment suggesting that the culture confirmed, but PCR negative sample, may have contained a low level of *S. enterica* contamination that was below the detection limits of the PCR (300 $CFUml^{-1}$). To confirm this, the *iroB* gene PCR would need to be applied to secondary enrichment cultures.

### 7.6.5 **Summary of findings from the IPC and PC assays**

The IPC and PC control PCR assays were not performed on the same samples, as the IPC was qualitative and only applicable to the PCR reaction and the PC was quantitative and applied the completed process. A direct comparison between the two methods was not performed; however, it may have been useful to apply both methods to any sample that was considered inhibited by one method as additional validation. Cost and approximate operator time of the two assays was similar.

Enrichment cultures for the detection of the Gram-positive target organisms were tested with the IPC control to monitor for inhibition in the PCR only. The PC control, in contrast to the IPC, offers the advantages of monitoring the DNA extraction and any loss of sensitivity throughout the assay. The PC control was only used initially as a control for Gram-negative organism. It was considered not to be a suitable DNA extraction control for Gram-positive organisms because they generally have a thicker peptidoglycan layer in their cell wall and thus often need additional cell lysis procedures (discussed in Chapter 4). However, in this study a cell lysis step using heat and proteinase K was found to release DNA from the Gram-negative organisms and the Gram-positive *L. monocytogenes*. A direct comparison of the two strategies would have been interesting especially in the detection of *L. monocytogenes* assays that were optimised to work with either type of control.

The work in this thesis used an *E. coli* expressing GFP as in process control. Klerks *et al.* (2006) also used an *E. coli* strain harbouring a plasmid expressing green fluorescent protein as a general internal control in an experiment to compare five commercially available DNA extraction methods for preparing *S. enterica* DNA from soil samples. As shown in the DNA extraction work presented here, these authors report that inhibiting components should be identified to allow the development of more generic DNA extraction methods for the preparation of DNA from more complex substrates. However in some instances (such as boiling) generic extraction approaches may be insufficient to prevent poor DNA recovery or the presence of co-extracted amplification inhibitors and these will be identified by the use of a process control in the extraction.

As the GFP originates from the jellyfish *Aequorea Victoria* (Chalfie *et al.,* 1994), a major advantage of using GFP as an PC is that it is not naturally present in the samples being tested and hence we can be sure that any detection in the sample only results from the lenticulated strain. This is an advantage over other previously published approaches that use house-keeping genes to control the DNA extraction and amplification efficiency, because the initial amount of control material in the sample is not known thus only allowing qualitative validation of the sample tested (Klerks *et al.,* 2006).

A GFP expressing *E. coli* O157:H7 and a *S. enterica* serovar Typhimurium were constructed and spiked on to lettuce to quantify contamination by detection of the green fluorescent protein (Noah *et al.,* 2005). These organisms were also used as internal controls for detection of *E. coli* O157:H7 and a *S. enterica* serovar Typhimurium by the detection of the green fluorescent protein (Noah *et al.,* 2005) and detection of the *gfp* gene (Klerks *et al.,* 2006) however the latter study was not applied to naturally-contaminated foods. The work presented here had the added advantages of producing the quantified lenticulated strain containing a chromosomally coded, and therefore more stable, lower copy number of the *gfp* gene that could be used for the detection of five different bacterial pathogens.

The assay, from receipt of food sample to PCR result, took approximately six hours to perform and this represents a considerable time saving compared with current microbiological identification and confirmation, which takes two days to produce an unconfirmed result. Confirmation of the results means that conventional microbiological assays would take an additional two days to produce a result. Reasons PCR assays have not been widely implemented in food microbiological testing laboratories include the cost of reagents and instrumentation, establishing molecular testing facilities and training of staff. However, if robust and reliable PCR techniques can be validated for the detection of pathogens in food, such as presented in this work, the benefits of a fast turnaround time and high throughput may override this obstacle (Mothershed and Whitney, 2006). The development of validated PCR assays with enrichment broths described in the gold standard culture methods facilitates the integration of the PCR method into routine diagnostic laboratories (Rossmanith *et al.,* 2006).

## 7.7 Conclusions

In conclusion, this work demonstrates that a rigorous nucleic acid preparation technique coupled with robust, sensitive and specific PCR assays should be combined with an internal process control strategy to provide a reliable strategy for the application of PCR for the detection of bacterial pathogens in enrichment broths inoculated with food. The control strategies, such as those described here, are important tools for the interpretation of PCR assays for the detection of pathogens in food.

The hypothesis of this study was if PCR assays can be designed or modified to be robust and reliable when applied to the detection of bacterial pathogens directly in food samples then they will offer a rapid alternative to cultural detection. The PCR assays were shown to be rapid and robust offering an alternative to the traditional culture-based methods of detecting the target pathogens in food samples if culture enrichment is employed. Implementation

of these PCR assays and control strategies in food microbiology laboratories will provide a high-throughput analysis that can be used alongside cultural analysis to screen out negatives and give more rapid indications of contaminated foods.

# CHAPTER 8

GENERAL DISCUSSION AND SUGGESTIONS FOR FUTURE WORK

## 8.1 General discussion - study aims and results obtained

The overall hypothesis that was tested in this thesis is whether PCR assays can be developed that are equal or better, than cultural detection in terms of robustness and reliability when applied to the detection of bacterial pathogens directly in food samples. Currently conventional culture-based methods are used for detection of bacterial pathogens in foods. These microbiological analyses are powerful and dependable but because of their length and cumbersomeness they are often not compatible with the speed at which food products are manufactured and the short shelf life of products (Bhunia, 2008). From a public health perspective, faster detection times are essential to prevent the spread of infectious disease and the identification of a continuing source of infection (Abubakar *et al.,* 2007).

PCR was identified as a rapid method of pathogen detection, however it has not been widely accepted for the application to contaminated food samples. The key issues that were hindering the use of PCR were identified in this study as that current PCR methods are subject to false positive and false negative results due to components of the food matrix and the volume of food required to be examined for low-level contamination. To address these challenges methods of bacteria DNA extraction and method internal control were investigated to reduce potential PCR false negatives. The optimised sample preparation was then used with real-time PCR assays and compared with the 'gold standard' culture analysis of food samples for bacterial contamination. The experiments and outcomes are explained in more detail in the next sections.

### 8.1.1 **Food sample preparation for PCR analysis**

MagNA Pure™ was investigated for extraction for DNA from contaminated food samples to give successful PCR amplification for the detection of bacterial pathogens. MagNA Pure™ extraction was compared to seven DNA

preparation techniques for the preparation of *L. monocytogenes* DNA and was shown to be advantageous. It is automated allowing for high throughput and it is reliable and robust when applied to complex food matrices (Chapter 3). This study was the first description of MagNA Pure™ for the preparation bacterial pathogens from contaminated food samples.

The MagNA Pure™ method was used to prepare DNA that was successfully amplified in a16S rDNA PCR, however unfortunately *S. aureus* DNA was possibly autodegraded by DNases that are abundant in these organisms and contribute to pathogenicity (Tang *et al.,* 2008). DNA extraction methods can cause false-negative PCR results, if they fail to extract sufficient target molecules from a contaminated sample or if PCR inhibitors are co-precipitated with the DNA (Lampel *et al.,* 2000). Food, as a sample medium, is highly variable in its constituents and so can present ever-changing problems for DNA extraction methods, this was overcome in part by choosing a complex DNA extraction and purification procedure, however it was noted that an internal control to monitor for failing of the extraction process was essential. The presence of PCR inhibitors co-precipitated from food samples was monitored in the reaction by using an internal positive control in the PCR reaction however, the assay could be further improved by also monitoring the extraction.

Optimisation of upstream processes for application to PCR rather than culture detection is required to improve the robustness of PCR assays. For example, stomaching of samples can release food constituents that cause PCR inhibition and *Salmonella* has been detected in a greater numbers from un-homogenised or manual massaged samples of minced chicken compared to stomached samples (Kanki *et al.,* 2009). Ki *et al.* (2007) described an automated magnetic bead system with a bore-mediated grinding system, which incorporated DNA purification. This technology is advantageous and rapid not requiring prior pre-treatment or disruption, which may allow preparation of bacteria from food samples without the need for the initial dilution step and cultural enrichment (Ki *et al.,* 2007).

Alternatively, a new technology that incorporates solubilisation of the food matrix using a protease prior to real-time PCR detection could be used for sample preparation (Mayrl *et al.,* 2009). This solublization assay was applied to *L. monocytogenes, S. aureus* and *Salmonella* spp. in artificially contaminated foods and was advantageous because results were obtained within five hours and the average detection limit was 7.3 CFU $ml^{-1}$ (Mayrl *et al.,* 2009).

### 8.1.2 **Internal positive process control (PC) development**

DNA extraction and PCR were monitored by detecting an added surrogate organism in the sample. The surrogate organism had to be a bacterial strain that can be differentiated from flora in the sample and so a novel *E. coli* strain was constructed carrying a *gfp* gene (Chapter 4). The *E. coli* GFP strain was encapsulated in a Lenticule disc that provided the control organism in a stable, quantitative and biologically active format. The addition of a known amount of target organism PCR allows for the loss of sensitivity to be calculated. A novel sensitive and specific 5' nuclease real-time PCR was developed to detect the *gfp* gene that was duplexed with pathogen gene targets. The employment of limited concentrations of the forward and reverse oligonucleotides in the duplex PCR assay allowed for the sensitive detection of the pathogens. This was developed and applied in the testing of naturally-contaminated food samples in the field trial.

### 8.1.3 **Detection of viable cells**

The main objective of this study was to develop molecular methods for the detection of foodborne bacterial pathogens as an alternative to culture detection. One of the advantages of culture detection is that the methods will only detect viable cells. To investigate a method that would also detect viable cells, mRNA was investigated as a molecular marker of the identifying presence of viable *L. monocytogenes* cells (Liu, 2008). RT real-time PCR and

NASBA were investigated as molecular methods that could be used to detect mRNA but were unsuccessful. The RT real-time PCR detected *L. monocytogenes* in samples that had been killed (by autoclaving) and were unculturable. Therefore, real-time RT-PCR applied in this study could result in false positive results.

NASBA assays were also investigated, but the *L. monocytogenes* mRNA target was not detected. Due to the failures of real-time RT-PCR and NASBA for the detection of mRNA, this approach was abandoned. EMA combined with PCR has shown utility as a method for the detection of viable *L. monocytogenes* in culture and could be applied to samples if direct detection (i.e. without culture enrichment) or molecular quantification was performed (Nocker *et al.,* 2008; Nocker *et al.,* 2006; Nogva *et al.,* 2003; Rudi *et al.,* 2005b). The method was not continued through to the field trial, as the PCR assays were not sufficiently sensitive when applied to food samples without prior enrichment culture.

### 8.1.4 **Development of PCR assays**

PCR was selected as the method to amplify and detect bacterial pathogens in food, as it is a sensitive, specific and rapid method. Using multiplex PCR numerous target genes can be amplified simultaneously creating a single screening assay that can be applied to contaminated food samples (Oh *et al.,* 2009). Bacterial pathogens comprising *C. coli, C. jejuni, C. perfringens, E. coli* O157:H7, *L. monocytogenes*, *S. enterica* and *S. aureus* were targeted for PCR detection, as they are most often associated with foodborne illness in the UK (Adak *et al.,* 2005). Previously published PCR assays were selected for the detection of *L. monocytogenes* (Nogva *et al.,* 2000), *C. perfringens* (Amar *et al.,* 2005) *C. coli* and *C. jejuni* (Best *et al.,* 2003). Novel real-time assays were designed for the detection of *E. coli* O157:H7, *S. enterica* and *S. aureus* (Chapter 6).

A fully nested assay was designed for the detection of *L. monocytogenes* and was shown to increase the sensitivity of the existing real-time PCR sensitivity from 1100 to 1.1 CFU/PCR. The nested assay was applied to CSF and serum from eight patient samples with culture confirmed listeriosis. In a further nine samples from patients where *L. monocytogenes* was not cultured but the patients presented clinical symptoms suggestive of listeriosis, *L. monocytogenes hlyA* was detected 6/9 when the nested assay was applied. The nested assay gave an increase in detection of the *L. monocytogenes hlyA* from 35% to 82% of the samples and only failed to detect the organism in one culture positive sample. Molecular methods of pathogen detection are useful when a rare or unexpected microorganisms for the sample type are present or the sample is collected during antibiotic treatment (Zaloudikova *et al.,* 2009).

To increase the sensitivity of the other real-time PCR assays a hemi-nested phase was designed for each of the existing real-time PCR assays described in this study. Hemi-nesting was chosen over fully nested as less oligonucleotides were required to be designed. As probes were used for detection, the increased specificity gained from using two oligonucleotides was not required. The hemi-nested assays used as a multiplex PCR and amplicons were detected using a suspension bead array. The development of the multiplex PCR was not fully completed, as it requires further optimisation to achieve detection of each amplicon type and then full validation of the method is required. Ultimately, the PCR assays were validated for real-time detection and shown to be sensitive and specific for the detection of their respective pathogens, and thus suitable for use in a field trial.

### 8.1.5 **Application of PCR assays to culture techniques for detection of bacterial pathogens in naturally-contaminated food samples**

Finally, the optimised assays were applied to samples for the detection of the target pathogens in naturally-contaminated food samples in a field study. This field trial used a combination of methods investigated in this work including conventional food sample preparation techniques, MagNA Pure™ DNA extraction, real-time PCR and two types of internal positive process control.

The results of PC PCR assays applied to detect *Salmonella* spp. and *L. monocytogenes* in the field trial and construction of the PC have been described in Murphy *et al.* (2007).

In total 506 enrichment cultures inoculated with food and environmental samples were tested for the presence of *E. coli* O157:H7, *C. jejuni, C. coli, L. monocytogenes* and *S. enterica* by real-time PCR. The results of the PCR assays were compared to those obtained by the F.W.E laboratory that tested the foods by culture methods. Molecular detection of any one of these bacterial targets was detected in 47 of the 506 samples tested (9%), 35 of which were confirmed by culture. Overall, a low proportion of contaminated samples were detected by either PCR or culture.

PCR was shown to be an ideal technique for the screening of target negative samples, since there was a high level of agreement between results obtained by conventional microbiology and PCR. Negative samples do not require analysis, and their elimination at an early stage would greatly reduce both the cost and time taken for pathogen detection and microbiological surveillance.

There were a small number of samples where the *iroB*, *hlyA, mapA* or *ceuE* gene fragments were detected by PCR, but the results were not confirmed by culture. These differences could be due to either technical laboratory error, non-specific amplification or cross contamination with target nucleic acid. However, duplicate test results were always concordant, PCR assays were demonstrated to be 100% specific for their bacterial targets when tested with a range of other bacterial species, and positive and negative controls were not consistent with either laboratory error or cross-contamination. Possible explanations for these PCR positive, culture negative results include either an increase in the sensitivity of PCR compared to culture, or the detection of target DNA from dead or sub-lethally injured organisms not recovered by conventional culture. Mafu *et al.* (2009) compared real-time PCR to culture for detection of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* on food contact surfaces and showed when surfaces were initially sampled there was a

correlation between PCR and culture analysis. However, after 16 hours detection was possible by PCR, but not by culture and concluded this was due to the superior sensitivity of the PCR (Mafu *et al.,* 2009).

While agreement was obtained between the PCR and conventional testing methods for the vast majority of samples, it should be noted that the majority of the PCR assays were only applied to primary enrichment cultures, whereas the cultural analyses use further enrichment procedures and sub-culture to selective agar, and therefore the results were not strictly directly comparable. It is feasible, that applying the PCR to secondary enrichment cultures or isolates from selective agars might lead to a further increased sensitivity. However, this has the disadvantage of considerably increasing the time taken to generate a result and hence these comparisons were not performed in this study, because the aim was to develop a rapid test that minimised the amount of traditional microbiology.

Ideally, this study would have completed the multiplex assay and used it in combination with EMA for detection of viable cells and with MagNA Pure™ and the process control organism (*E. coli* GFP), however, it would have required extensive further work on the sample preparation method that was outside of the timeframe of this study. The PCR detection methods described here require further refinement to improve them and enable them to be multiplexed and applicable to improving the food sample preparation and cultural enrichment stage. This may also include the development of novel extraction procedures.

The majority of published PCR detection assays applied to detect bacterial pathogens in food samples require cultural enrichment prior to detection for sufficient sensitivity (Jiang *et al.,* 1998). However, enrichment broths tend to be pathogen specific and so not compatible with multiplex detection methods. An alternative to specific enrichment is universal enrichment broth, which has been developed for the simultaneous recovery of several pathogens types, for example, *E. coli*, *Salmonella* spp. and *L. monocytogenes* (Jiang *et al.,* 1998). A

selective broth for the simultaneous culture of *S. enterica*, *E. coli* O157:H7 and *L. monocytogenes* was found to be comparable to universal enrichment broth and inhibited a greater number of non-target organisms (Kim and Bhunia, 2008). A consideration of using universal enrichment broth is the recovery of damaged cells which may need additional nutrients or culture conditions to allow recovery and growth (Donnelly, 2002; Gnanou *et al.,* 2000; Gracias and McKillip, 2004). Cultural analysis has shown that some *L. monocytogenes* have low recovery using current techniques (ISO 11290-1) needing up to 144 hours to obtain 95% recovery (Leclercq, 2004). Thus shortening enrichment times could result in false-negative results.

Decreasing or eliminating the need for enrichment would allow the full benefits of molecular detection methods to be realised (Stevens and Jaykus, 2004). The direct detection and quantification of bacteria in foods using real-time PCR has been described and compared to most probable number culture methods (Guy *et al.,* 2006; Malorny *et al.,* 2008; Wolffs *et al.,* 2005). Methods that separate the microorganisms from the food sample and concentrate them in to a smaller volume have been suggested to increase the sensitivity of many rapid detection techniques and are an alternative to the lengthy process of cultural enrichment (Wilson, 1997). Concentration and separation techniques applied to bacterial cells from complex sample matrices have been reviewed by Stevens and Jaykus (2004). For example, zirconium hydroxide was used to immobilise and concentrate human enteric viruses which included hepatitis A virus and Norwalk virus from clinical, environmental and food samples for PCR amplification and detection (D'Souza and Jaykus, 2002). Immunomagnetic separation has been used in combination with PCR the sensitive and simultaneous detection for *L. monocytogenes* and *Salmonella* spp. in foods (Hsih and Tsen, 2001; Li *et al.,* 2000). Real-time PCR has also combined with concentrating the sample by filtration rather than using cultural enrichment for the detection of bacteria in slaughterhouse swabs (Rueckert and Morgan, 2007), however PCR quantification was not consistent with viable counts for enumeration of coliform bacteria (Guy *et al.,* 2006).

Further advances in bacterial capture technology include the solid capture of bacteria using ganglisosides for immobilising *S. enterica, E. coli* and Bacillus spores prior to real-time PCR and improved detection limits 100-fold (Desai *et al* 2008). However, in the presence of competing organisms the lowest level of detection was 4 x $10^4$ $CFUg^{-1}$ (Desai *et al.,* 2008). The application of DNA aptamers, single stranded oligonucleotides that naturally fold into three dimensional structures and have the capacity to specifically bind to biosurfaces, has been used for the capture and detection of *Salmonella* (Joshi *et al.,* 2009) . The DNA aptamers were bound to magnetic particles and used to separate *S. enterica* serovar Typhimurium from spiked whole chicken carcass rinses and detection with real-time PCR detection limits were $10^2$ to $10^3$ CFU per 25g (Joshi *et al.,* 2009). Novel cationic charged magnetic particles with automated capture, that re-circulates the sample homogenate has also been used to concentrate hepatitis A virus from artificially contaminated food samples prior to detection by RT-PCR. The detection limits were approximately $10^2$ plaque forming units per 25g (Papafragkou *et al.,* 2007). An significant advantage of these systems is that large volumes can be analysed and the sample is concentrated up to 500-fold (Papafragkou *et al.,* 2007).

## 8.2 Beyond this study- suggestions for future work

### 8.2.1 New technologies for detecting bacterial pathogens in foods

New technologies may present superior, alternative methods for detecting foodborne pathogens. Examples of these technologies that have been applied to food samples include quantum dot (Bruno *et al.,* 2009) novel light scattering sensors (Banada *et al.,* 2009) and electrochemical impedance detection techniques (Yang and Bashir, 2008). Methods that utilize biosensors, which are devices that combine detection of an analyte with a biological component with a physicochemical detector component, have been applied for the detection of foodborne pathogens. Examples of mass-based biosensor methods applied for detection of foodborne pathogens have been reviewed by Bhunia (2008) and electroanalytical biosensors have been reviewed by Palchetti and

Mascini (2008). Biosensor technology has the potential to speed up detection, increase specificity and sensitivity and enable high-throughput analysis (Palchetti and Mascini, 2008).

Biosensor technology is in its infancy when applied to complex naturally-contaminated food samples and will require considerable optimisation before it can be accepted as a reliable detection method for pathogens in the food industry. However, biosensor methods may be more readily accepted if combined with existing technologies, for example, Marques *et al.* (2009) described a method using an electrochemical biosensor with thiolated PCR amplicons for the detection of *Salmonella* spp. which was sensitive and could detect 9 fmol of DNA.

Alternative new technologies, for example, capillary electrophoresis-based single-strand conformational polymorphism have also been coupled with multiplex PCR and this method has been reported to allow the simultaneous detection of eight foodborne bacterial pathogens (Oh *et al.,* 2009). More tradition methods such as filtration have also been combined with PCR to provide novel assays that have the potential to detect and quantify viable foodborne pathogens (D'Urso *et al.,* 2009). Advancements in hybridisation technology have led to the development of DNA microarrays that enable the detection of many DNA target sequences. The use of DNA microarrays has been employed to increase the speed and specificity of detection of bacterial species present in a sample (Ye *et al.,* 2001). DNA microarray combined with multiplex PCR has been shown to simultaneously detect 34 virulence genes from the following bacteria: *E. coli*, *V. cholera, V. parahaemolyticus, S. enterica, C. jejuni, Shigellae, Y. enterocolitica* and *L. monocytogenes* in stool specimens (You *et al.,* 2008). This tool may play a major part in future molecular-based pathogen detection assays.

### 8.2.2 **Food safety on a global scale**

Beyond identification of a contaminated food product, it is essential to perform typing of pathogens and epidemiological studies to track food poisoning outbreaks and for proactive food safety assurance. For example, surveillance methods, including Salm-net (a network for the surveillance of salmonellosis in Europe), were used to identify an outbreak of *S. enterica* Agona in peanut snacks (Killalea *et al.,* 1996). Cases were subsequently identified in the UK, Israel and North America (Killalea *et al.,* 1996). Travel and trade have increased over the years, increasing the risk of dissemination of pathogens (Malorny *et al.,* 2003b). International investigations of outbreaks of foodborne disease have shown that with the globalisation of food production and trade, pathogens are more likely to cross national borders and a single meal can contain products from many countries (Tauxe and Hughes, 1996). It is necessary to consider food contamination and foodborne disease as a global concern with a harmonised approach to monitor and control the problem to ensure the supply of food that is safe to eat.

### 8.2.3 **The future predictions for microbiological food safety**

PCR detection methods are now being used in food microbiology laboratories (Malorny *et al.,* 2008). The routine application of PCR to naturally-contaminated food samples will result in increased knowledge of bacterial food contamination events, with further standardisation and validation producing improved assays with greater utility. Therefore, new technologies may present superior, alternative methods to PCR or culture for detecting foodborne pathogens. When developing detection methods we must consider potential change of food contamination risk indicated by communicable disease surveillance, possibly from the less common bacterial and non-bacterial foodborne pathogens for example, virus and parasites, and the recognition new or emerging pathogens.

The safety of our food supply will remain a major concern to consumers, food producers, and regulatory agencies (Bhunia, 2008) and has recently been given priority by UK research councils (see BBSRC.AC.UK). Food safety from farm-to-fork through the supply chain must be maintained to protect consumers from debilitating and sometimes fatal episodes of pathogen outbreaks (Bhunia, 2008). Ultimately, it is a combined approach of rapid, sensitive and accurate detection methods integrated with HACCP, food safety education with continued vigilance and surveillance that is applied on a global-scale, which will ensure the safety of our food supply.

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## APPENDIX 1

**Figure 8.1 Partial *L. monocytogenes hlyA* gene alignment with NASBA oligonucleotide and probe positions in Genbank AL591977**

### Blais *et al.*, (1997) method

260 TTTCATCCAT GGCACCACCA GCATCTCCGC CTGCAAGTCC TAAGACGCCA
310 ATCGAAAAGA AACACGCGGA TGAAATCGAT AAGTATATAC AAGGATTGGA
360 TTAYAATAAA AACAATGTAT TAGTATACCA CGGAGAYGCA GTGACAAATG
410 TGCCGCCAAG AAAAGGTTAY AAAGATGGAA ATGAATATAT YGTTGTGGAG

### NASBA assay designed in this study

2360 ATCCTCCTGC ATATATCTCA AGTGTGGCGT ATGGCCGTCA AGTTTATTTG
2410 AAATTATCAA CTAATTCCCA TAGTACTAAA GTAAAAGCTG CTTTTGATGC
2460 TGCCGTAAGC GGAAAATCTG TCTCAGGTGA TGTAGAACTA ACAAATATCA
2510 TCAAAAATTC TTCCTTCAAA GCCGTAATTT ACGGAGGTTC CGCAAAAGAT
2560 GAAGTTCAAA TCATCGACGG CAACCTCGGA GACTTACGCG ATATTTTGAA

The oligonucleotide and probe target are highlighted as follows: BTP1, BTP2, NASBAFWD, NASBABeacon, NASBAREV.

## Figure 8.2 Oligonucleotide and probe positions for PCR and Bio-plex

M62654 *Aequorea victoria* (*gfp*)

541 ACTAGCAGAC CATTATCAAC AAAATACTCC AATTGGCGAT GGCCCTGTCC TTACCAGA
601 CAACCATTAC CTGTCCACAC AATCTGCCCT TTCCAAAGAT CCCAACGAAA AGAGAGATCA
661 CATGATCCTT CTTGAGTTTG TAACAGCTGC TGGGATTACA CATGGCATGG ATGAACTATA

X88849 *Campylobacter coli* (*ceuE*)

3241 ATTGCAGATA TTAAAAATGA GATAGAACAA GCAAAAAGCA TAGTAGATGA AGATAAAAAA
3301 GCTCTTATTG TTCTAACCAA TTCTAACAAA ATTTCCGCTT TTGGACCTCA ATCTCGCTTT
3361 GGAATCATTC ATGATGTTTT AGGAATCAAT GCTGTGGATG AAAATGTAAA AGTAGGCACA
3421 CATGGAAAAA GCATTAATTC TGAATTTATA CTAGAAAAAA ATCCTGATTA TCTATTTGTA
3481 GTTGATAGAA ATATCATTGT GGGCAATAAA GAACGCGCAC AAGGCATACT TGATAATGCA

X80135 *Campylobacter jejuni* (*mapA*)

301 AAAGTCCTGG TGGTTTTGAA GCAAAGATTA AAGGGCTTTT ATACATTAGC GATGTTGGAA
361 TTCAATGTTG TGCCAATAAA CGCACTTTAG ACACTGGTAT TGCTTTGAAA AAGGTTTATT
421 TACATAGATT TTATGATTTA AAAGAAGGGC AAAAGGTTTT AAATGCTAAA GGGAAAAAGT
481 TATTTGTCGA TGTGAATTTT AATGCGGTAT TTTATACTTA TTTAAAACAA GAACTTGAAG
541 CTAGAGGAAT AGTTGTGCTT GACAATAACG ATCAAAATTC ACCTTATGTG AGTAAGATTG

M98037 *Clostridium perfringens* (*cpe*)

961 GGTAATTTAT ATGATTGGCG TTCTTCTAAC TCATACCCTT GGACTCAAAA GCTTAATTTA
1021 CACTTAACAA TTACAGCTAC TGGACAAAAA TATAGAATCT TAGCTAGCAA AATTGTTGAT
1081 TTTAATATTT ATTCAAATAA TTTTAATAAT CTAGTGAAAT TAGAACAGTC CTTAGGTGAT
1141 GGAGTAAAAG ATCATTATGT TGATATAAGC TTAGATGCTG GACAATATGT TCTTGTAATG

M24904 *Clostridium perfringens* (*cpa*)

481 GGGAATCACA AATAAGAAAA TTTTCAGCAT TAGCTAGATA TGAATGGCAA AGAGGAAACT
541 ATAAACAAGC TACATTCTAT CTTGGAGAGG CTATGCACTA TTTTGGAGAT ATAGATACTC
601 CATATCATCC TGCTAATGTT ACTGCCGTTG ATAGCGCAGG ACATGTTAAG TTTGAGACTT

**Figure 8.2 cont. Oligonucleotide and probe positions for PCR and Bio-plex**

AF081182 *Escherichia coli* (*eaeγ*)

2521 TATATGATAA AAGTGGATAA GCAAGCCTAT TATGCTGATG CTATGTCCAT TTGCAAAAAT
2581 TTATTACCAT CCACACAGAC GGTATTGTCA GATATTTATG ACTCATGGGG GGCTGCAAAT
2641 AAATATAGCC ATTATAGTTC TATGAACTCA ATAACTGCTT GGATTAAACA GACATCTAGT
2701 GAGCAGCGTT CTGGAGTATC AAGCACTTAT AACCTAATAA CACAAAACCC TCTTCCTGGG

AY174657 *Listeria monocytogenes* (*hlyA*)

241 AAAATTCAAT TTCATCCATG GCACCACCAG CATCTCCGCC TGCAAGTCCT AAGACGCCAA
301 TCGAAAAGAA ACACGCGGAT GAAATCGATA AGTATATACA AGGA**TT**GGAT TACAATAAAA
361 ACAATGTATT AGTATACCAC GGAGATGCAG TGACAAATGT GCCGCCAAGA AAAGGTTACA

BX571856 *Staphylococcus aureus (nuc)*

896101 TGCAAAGAAA ATTGAAGTCG AGTTTGACAA AGGTCAAAGA ACTGATAAAT ATGGACGTGG
896161 CTTAGCGTAT ATTTATGCTG ATGGAAAAAT GGTAAACGAA GCTTTAGTTC GTCAAGGCTT
896221 GGCTAAAGTT GCTTATGTTT ATAAACCTAA CAATACACAT GAACAACTTT TAAGAAAAAG
896281 TGAAGCACAA GCGAAAAAAG AGAAATTAAA TATTTGGAGC GAAGACAACGCTGATTCAGG
896341 TCAATAATGC TCATTGTAAA AGTGTCACTG CTGCTAGTGG CACTTTTATA ATTTTTAGAT

U62129 *Salmonella typhi* (*iroB*)

421 GGCGTGACGA AATCACTTTC TAACGCCTAC CGCCGCCATG GGGTCAGCGC GCCACCAAGA
481 GATCTGGCGT GGATAGACGT CACACCGCCC AGCATGAGCA TACTGCAAAA TGACGGAGAG
541 CCGGTTATCT CCATGCAATA CGTCCCGTAT AACGGCGGCG CCGTCTGGGA AGAATGGTGG

Titles provide Genbank reference number, followed by name of the organism and gene target in parenthesis.

The oligonucleotide and probe target are highlighted as follows: Forward oligonucleotide; Reverse oligonucleotide; Reverse external oligonucleotide; TaqMan probe (underlined); Bio-plex probe;

Forward external oligonucleotide for *L. monocytogenes* nested assay and Reverse altered oligonucleotide binding site for *L. monocytogenes* RT-PCR (mismatches shown in bold).