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The Use of Polymerase Chain Reaction in the Detection of Reoccurring Salmonella in Frozen Food Samples from Local Stores in Abeokuta Nigeria

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Abstract

Food-borne salmonellosis has been identified as one of the major causes of gastroenteritis worldwide. The need for more rapid and accurate techniques for the detection of food-borne pathogens has led to the development of novel molecular techniques. Though the conventional culture technique still stands as the golden standard for pathogen detection, it has proved to be laborious and takes 3 to 7 days to get a positive result. Rapid molecular techniques such as polymerase chain reaction (PCR) have been developed to produce more specific, sensitive, and accurate results within a shorter period. This study was performed to detect the presence of *Salmonella* in beef, pork, egg, prawn and chicken samples purchased from retail stores in Abeokuta Nigeria. The presence of *Salmonella* was assayed using the conventional culture technique and PCR with *Salmonella* Enteritidis primers specific to the *sefB* gene. Both culture and PCR were unable to detect *Salmonella* from the food samples. Probable reasons for this were discussed.

Key worss: *Food safety, Pathogens, Salmonella spp. Cultural methods, Polymerase chain reaction.*

Introduction

Food being an essential component of human life, could serve as an important and common source of infection. Different food samples serve as vectors for the acquisition and transmission of infectious diseases caused by several food-borne pathogens. Common food-borne pathogens include; *Escherichia coli, Salmonella, Campylobacter jejuni, Shigella, Listeria monocytogenes, Vibrio cholera, Hepatitis A Clostidium botulinum, Rotavirus, Norovirus, Ascaris lumbricoides, Toxoplasma gondii* and so on. Most food-borne infections are as a result of microorganisms that infect the living plant or animal or contaminate food or water during handling or processing (Sine, 1995; Todar, 2005). Food-borne infections remain a great concern of public health, as food and water contaminated by sources such as bacteria, toxins, parasites, viruses, plants, animal tissues, moulds, algae and chemicals can easily be encountered when proper sanitary measures are not put in place (Nantel, 1996). When these pathogenic organisms or toxic substances are ingested in food or water, they invade the intestinal mucosa and thereby multiply and invade host cells.

Due to the increased interest and awareness of the severity of food-borne diseases, the demand for monitoring pathogens in food samples in the shortest possible time has increased significantly (Riyaz-ul-Hassan *et al.*, 2004). The traditional culture method used to detect microorganisms from food samples usually involve pre-enrichment, selective enrichment, isolation on selective media and confirmation of the presumptive positive isolates by biochemical and serological tests (Fratamico, 2003; Hong *et al.*, 2003). The importance of enrichment cultivation is to accelerate the entire analytical process. Pre-enrichment could be done using non-selective enrichment broths such as buffered peptone water (BPW), maximum recovery diluent (MRD), nutrient broth (NB) or universal pre-enrichment broth (UPB) (Tsakila *et al.*, 2012). A distinctive feature of these non-selective broths is that they contain peptones as their source of minerals, carbon, nitrogen and vitamins. Although the presence of phosphates and sodium chloride in UPW and BPW also help maintain their buffering capacity and osmotic balance respectively.

These non-selective broths permit the growth of various organisms, which can be a form of setback when trying to isolate a particular organism. This drawback is made up for by the next step which is selective enrichment. Unlike pre-enrichment, selective enrichment is carried out using an enrichment medium that selects for and favours growth of the target organism. Series of enrichment media have been developed and appraised for the selective isolation of various pathogenic microorganisms. Examples include; Bolton broth (Oxoid microbiology products, 2012) for *Campylobacter jejuni*, buffered *Listeria* selective broth (Oxoid microbiological products, 2012) for *Listeria monocytogenes* and rappaport vassiliadis soy broth for the selective isolation of *Salmonella* (Hein *et al.*, 2006; Taskila *et al.*, 2012). After the process of pre-enrichment and selective enrichment, the concluding steps (isolation on selective media and confirmation of the presumptive positive isolates by biochemical and serological tests) are very much specific and often tailored to suit the target microorganisms. With the increase in food-borne infections came an increase in the demand for faster, safer and more efficient means of isolating and identifying infectious pathogens. Over the years there have an improvement from traditional culture techniques to rapid

molecular techniques such as; polymerase chain reaction (PCR), real time PCR, enzyme-linked immunosorbent assay (ELISA), gel electrophoresis, micro-array, restriction fragment length polymorphism (RFLP), macromolecule blotting and probing (Hein *et al.*, 2006; Almeida *et al.*, 2010; Cheung *et al.*, 2012; Taskila *et al.*, 2012). Mark and Warren (2003) illustrate that the impelling force for the development of new diagnostic techniques came from the escalating

Recent studies have focused on the need for more rapid and less laborious means of detecting food pathogens, especially due to the perishable nature of food and food products which are the vectors for food-borne pathogenic microorganisms (Saroj *et al.*, 2008).

These new methods not only have to be rapid but also need to be specific and highly sensitive (Seo *et al.*, 2003; Malorny *et al.*, 2007; Saroj *et al.*, 2008; McGuinness *et al.*, 2009). As far as molecular biology technology is concerned, the study of rapid molecular diagnostic techniques is an important aspect of molecular medicine (Riyaz-Ul-Hassan *et al.*, 2004), with several researches concentrating on how to perfect existing techniques and also the discovery of novel ones (Hassanein *et al.*, 2011). However the increasing interest in the reproducibility and accuracy of molecular techniques such as PCR has heightened the need for more research to be carried out under different conditions and varying protocols. PCR has appeared more prominent and widely studied due to its combination of simplicity accompanied by accuracy, specificity, and sensitivity. Different research are being carried out, focusing on the specific and most conducive experimental conditions suitable for various food pathogens concerning the food samples and the inhibitory effect of components present in different food types (Hassanein *et al.*, 2011; Saroj *et al.*, 2008;). As PCR is one of the most prominent molecular techniques, some food-borne pathogens have a higher prevalence than other pathogens. *Salmonella* is one of the most frequent causes of food-borne gastrointestinal infection worldwide that can be transmitted either between humans or from animals to humans (Agarwal *et al.*, 2002; Wang *et al.*, 2009; *Kusar et al.*, 2010; Hassanein *et al.*, 2011). In light of this, the purpose of this investigation is to identify the presence of *Salmonella* in various food samples using both traditional culture method and PCR. It would be carried out with the aim of analysing how recent developments in the field of molecular biology have led to more reliable identification and surveillance of *Salmonella* in food.

Materials And Method

Food samples

The following food samples were used for the isolation of *Salmonella*: frozen chicken, unpasteurized milk, cheese produced from unpasteurized milk, pork, egg and beef. Food samples were purchased from various local stores in Abeokuta Nigeria.

Isolation and identification of Salmonella using the conventional culture technique

This procedure was carried out using the standard culture procedure of pre-enrichment, selective enrichment, selective plating on solid media and biochemical analysis of presumptive colonies (Fratamico, 2003; Hong *et al.*, 2003).

Pre-enrichment

The samples used in this present study have been listed above in section 3.2. These food samples were all purchased within their period of expiry, in appropriate holding conditions and processed on the day of purchase.

The different food types were tested for the presence of *Salmonella* spp. by enrichment of 10 g or 10 ml (in the case of egg and milk) in 90 ml of Maximum Recovery Diluent (MRD; Oxoid, UK). Each 10 g or 10 ml of the samples were removed aseptically and added to different 100 ml volume flasks containing 90 ml of MRD and were incubated at 37ºC for 22 hours.

Selective enrichment

The secondary enrichment which was aimed at favouring the growth of *Salmonella* over all other food pathogens that might be present was carried out by adding 0.1 ml of the previously incubated MRD enrichment to 10 ml of Rappaport Vassiliadis enrichment broth (RV; Oxoid, UK) in glass universal bottles. The bottles were incubated at 42ºC for 24 hours.

Detection and isolation of Salmonella spp. on selective media

Serial dilution

Before plating on selective media, a 10-fold serial dilution was carried out on the incubated RV enrichment. Each sample bottle containing the incubated RV medium (the stock) was provided with 6 tubes (labeled 1 to 6), containing 9 ml of MRD each. 1 ml of the stock culture was added to tube 1, thereby producing 10 ml of the dilute solution. This process was repeated in succession for the remaining tubes with 1ml of the previous dilution added to the next tube. Each step results in a further 10-fold change in the bacterial concentration from the previous

concentration. Serial dilution was carried out to help reduce the bacterial colonies that would grow on the agar plate and thereby facilitate the counting process.

Selective plating

Dilutions of RV broth ranging between 10^{-3} to 10^{-6} were streaked onto Xylose Lysine Deoxycholate (XLD) agar plates with the use of a sterile inoculating loop. The plates were incubated at 37ºC for 24 hours. A pure culture of *S.* Typhimurium was used as the positive control. Due to the presence of very few colonies, another set of experiments was carried out without performing serial dilution. The RV broth was directly inoculated on the solid agar. Observed colonies were further analysed by gram stain and the applicable biochemical tests.

Gram stain

Gram stain was carried out on the bacterial isolates from the agar plates so as to differentiate these organisms into gram negative or gram positive on the basis of their chemical and physical cell wall property. A loop full of distilled water was placed on a clean microscope slide. Part of a colony was picked up using a sterile loop and homogenised in the distilled water on the slide, to create a smear.

The smear was then heat fixed by passing the slide over the Bunsen flame for 10 to 15 minutes. After heat fixing, the slides were placed on a rack and crystal violet was poured over the slides and left there for 60 seconds. The slides were washed with water and then flooded with iodine for 30 seconds. Iodine serves as a mordant which binds crystal violet and is therefore retained by gram positive bacteria. The slide was then washed again with water and flooded with ethanol which serves as a decolouriser, thereby removing crystal violet from gram negative cells. Safranin which serves as a counter stain was then applied to the slides and left there for 30 seconds. The slides were carefully blotted dry and observed under 100 x oil immersion lenses. Gram positive bacteria retain crystal violet and appear purple while gram negative bacteria retain safranin in their cell wall and appear pink when viewed under the microscope.

Biochemical analysis

Catalase test

A small portion of the test organism was put on a clean glass slide to which 1 to 2 drops of 3% hydrogen peroxide was added. The rapid appearance of continual gas bubbles indicates a positive result, while the absence of bubbles indicates a negative result. The production of bubbles is as a result of an enzyme which converts hydrogen peroxide to oxygen and water.

Molecular identification of Salmonella by PCR

DNA extraction

DNeasy® blood and tissue kit (Qiagen)

Pre-enrichment and selective enrichment was carried out as described in section 3.3 on fresh samples to be used for PCR. 5 ml of RV was centrifuged at 5000 x g for 10 minutes at 4ºC. DNA extraction was performed on the cell pellet using the DNeasy® Blood and Tissue Kit (Qiagen) following the manufacturer's instruction.

Phenol chloroform extraction

An alternative DNA extraction technique using phenol-chloroform was performed (Oliveira *et al.*, 2002). 5 ml of RV was placed in the microcentrifuge (Biofuge 13) at 3300 rpm for 5 mins. The supernatant was discarded and the pellets were re-suspended in 200 µL of lysis buffer and 20 µL of proteinase K. The mixture was incubated at 100ºC for 10 minutes, after which 150 µL of 3.0 M of sodium acetate was added and kept at -20ºC for 10 minutes. The resulting mixture was centrifuged at maximum speed for 10 minutes. The pellets were discarded and 680 µL of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma) was added to the supernatant. The Eppendorf tubes were mixed gently by inverting the tubes and this resulted in the development two phases; the lower organic phase and upper aqueous phase. This was centrifuged at 3300 rpm to further separate the solution into phases. The top phase containing the DNA was carefully removed into new tubes using 1000 µL pipette. 680 µL of chloroform/isoamyl alcohol (24:1) (Sigma) was added to it and mixed gently for 2 minutes. The mixture was centrifuged for 5 minutes at maximum speed (the chloroform would further separate the DNA to the top phase). The top phase containing the DNA was carefully removed using a 1000 µL pipette and transferred to a new tube. The DNA was precipitated by adding 360 µL of isopropanol and allowed to stand at room temperature for 10 minutes. The mixture was centrifuged for 10 minutes at 3300 rpm to pellet the DNA. The supernatant was discarded and the DNA pellets were washed with 70% ethanol. Mixing of the tube content was avoided and it was centrifuged at 3300 rpm for 10 minutes. The supernatant was discarded and the pellets were allowed to air dry. On drying completely, the pellets were re-suspended in 20-5 0µL of ultrapure water or TE buffer. This final mixture was stored in the freezer and to be used as a template for PCR.

Spectrophotometry readings using Nano-Drop

The Nano-Drop 1000 (Thermo scientific) machine was used to measure the amount and the purity of the DNA present in the purified DNA extract. Using sterile tips, 2 µL of distilled water was dispensed on the reading platform to blank the Nano-Drop. The Nano-Drop was then wiped clean and 2 µL aliquot of the purified DNA

extract for each sample was dispensed on the reading surface and the sampling arm was closed to initiate spectral measurement with the aid of the operating software on the PC. Each concentration was recorded.

Agarose gel electrophoresis

After nucleic acid quantification, agarose gel electrophoresis was performed on the chromosomal DNA extracted from the RV broth, so as to determine the presence as well as the size of the DNA. Using sterile pipette tips, 1 µL of the loading dye (6X) was added to 6 µL of the ladder (GeneRuler™, 100bp) and each sample which was then dispensed into different wells. The order in which the samples were loaded into the wells was recorded. The gel tank was closed by placing the lid in such a way that the wells are at the negative electrode. Since DNA is negatively charged, it runs from the negative electrode to the positive. The electrodes were connected to the power supply which was set to run at constantly 70 volts.

Primers and PCR conditions

The PCR mixture for the amplification of the *sefB* gene of *Salmonella enteric* serovar Enteritidis contained the 20nt (nucleotide) of forward and backward primers (Eurofins, UK). The primers; SefBF (5'-AGATTGGGCACTACACGT-3') and SefBR (5'-TGTACTCCACCAGGTAATTG-3') where expected to produce a DNA fragment of 535 bp (Wang *et al.*, 2009). The reaction mixture consisted of 5µL of the template DNA, 1 unit (0.2µL) of Taq polymerase (Sigma-Aldrich) 5µL of 10mM dNTP mix (Invitrogen) which already contains dATP, dCTP, dGTP, and dTTP in water (pH 7.5) each at a concentration of 10mM, 10µL of 5x reaction buffer and 1ml of each primer (1µM) in a final volume of 50µL. *S.* Typhimurium served as the template DNA for the positive control, while the negative control was void of any template. Amplification reactions were performed in a thermal cycler (BIO RAD) with the following thermal cycling protocol; DNA was denatured at 94ºC for 2 minutes and amplified for 35 cycles at 94ºC for 2 minutes, 60ºC for 50 seconds and 72ºC for 50 seconds. The final extension incubation was at 72ºC for 2 minutes. The PCR product was loaded on a 0.7% agarose gel and electrophoresis was carried out in 1 x TAE buffer at 70 volts. The gel was visualised in a UV box (Syngene) with the aid of the operating software on the PC.

Results

Table 1, details the observed outcome for the plating of Rappaport Vassiliadis enrichment broth on solid media as well as gram stain.

Table 1. Result for plating on solid agar, gram stain and catalase test of the observed colonies.

Sample	Media	Colony morphology	Colony number	Gram stain	Catalase test
Yoke	XLD	Large, raised, golden yellow colonies	8	Gram negative short rods	Positive
	MacConkey	Large, raised, pink colonies	3	Gram negative rods	
Beef	XLD	large, distinct, flat yellow colonies	55	Gram negative rods	Positive
	MacConkey	Large deep pink colony	1	Gram negative rod	
Prawn	XLD	Small red colony	1	Gram positive diplococci	Positive
	MacConkey	Red, minute colonies	30	Gram positive diplococci in clusters	
Pork	XLD	Small, distinct cream colonies	39	Gram negative rods	Positive
	MacConkey	Flat, distinct pink colonies	33	Gram negative short rods (coccobacilli)	
Milk	XLD	_	_	_	_
	MacConkey		_	_	_
Cheese	XLD	_	_	_	_
	MacConkey	_	_	_	_
Albumen	XLD	_	_	_	_
Albumen	MacConkey	_	_	_	_

DNA extraction and nucleic acid quantification

Table 2 shows the results obtained from both DNA extraction methods indicating that a higher concentration of nucleic acid was derived using the phenol-chloroform extraction.

Table 2. Comparison of the nucleic acid concentrations of DNA extracted from food samples using DNeasy® Blood and Tissue Kit (Qiagen) and phenol-chloroform extraction method

Samples	**Nucleic acid concentration (ng/µl)**	
	DNeasy kit	**Phenol-chloroform extraction**
S. Typhimurium (Positive Control)	31.1	5332.8
Egg	6.5	23.4
Beef	5.6	131.2
Prawn	15.6	_
Pork	8.8	476.9
Milk	4.8	_
Cheese	4.7	22.0

Specificity testing of the sefB-specific PCR assay

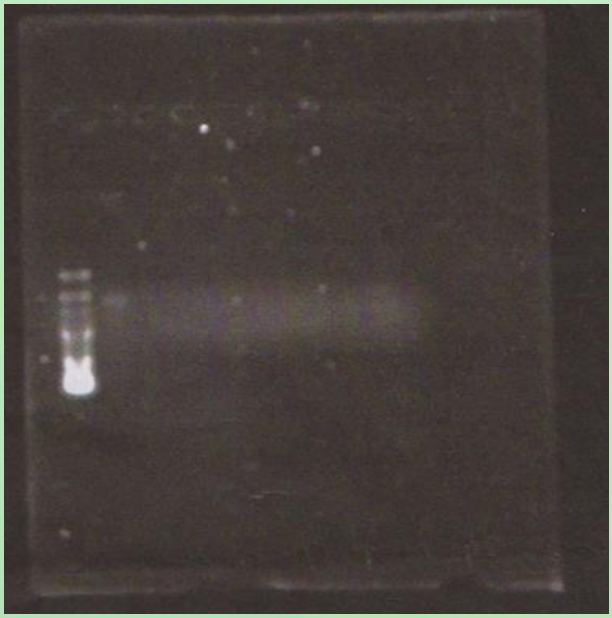

Figure 1. Gel electrophoresis of PCR product for the detection of Salmonella in food samples.
No visible bands were seen for the positive control and all samples (beef, pork, cheese, milk, egg and prawn). The first well contains the 100bp ladder.

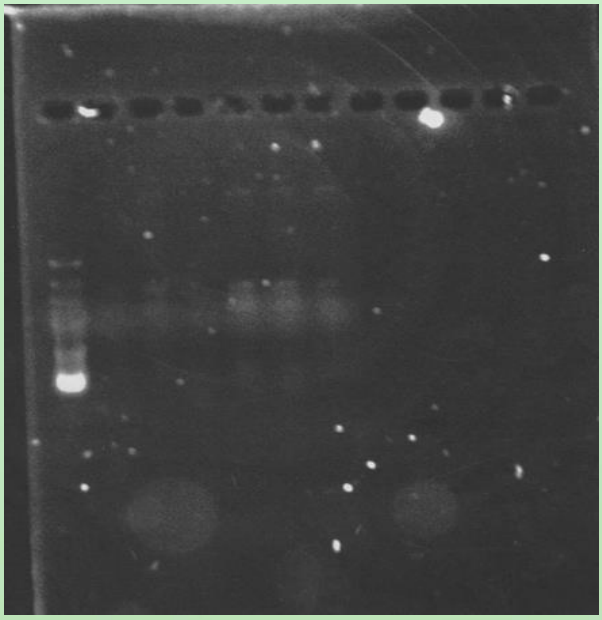

Figure 2. Gel electrophoresis of the rerun PCR assay for the detection of Salmonella in the same food samples.
No specific bands were seen for the positive control and all the food samples. Well 1 contains the gene ladder.

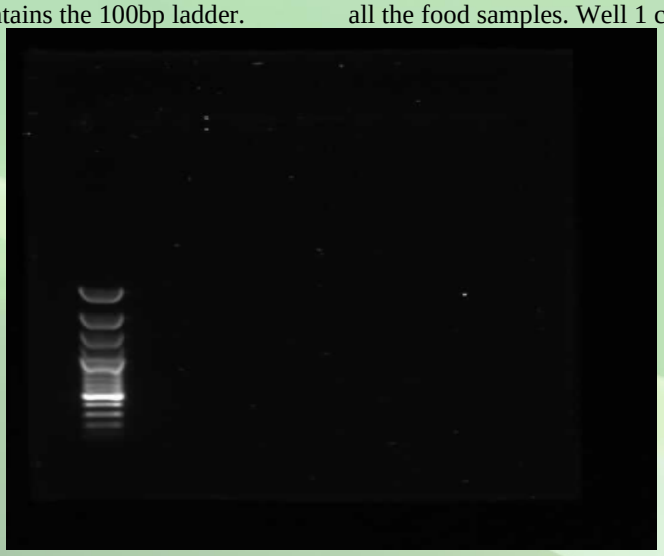

Figure 3. Gel electrophoresis of the PCR assay carried out at 50ºC annealing temperature. No visible bands were seen for the positive control and all samples. Well 1 contains the gene ladder.

Discussion

Conventional culture methods are identified as the basic and standard method of detecting pathogens such as *Salmonella* in food samples. Whyte *et al.* (2002), Hein *et al.* (2006), Wang *et al.* (2009) and Cheung *et al.* (2012) in their studies have shown that it would take 4 to 7 days to isolate and confirm the presence of these pathogens in food via standard culture methods. The purpose of this investigation was to carry out a comparative study which would further confirm the increased level of specificity and sensitivity associated with rapid molecular techniques such as PCR over the conventional culture methods. The enrichment protocol used involved the cultivation of *Salmonella* in MRD to facilitate the recovery of injured cells and then selective enrichment cultivation in RV broth. Studies have shown that a higher amount of *Salmonella* is recovered from food samples when RV enrichment is used (Fratamico, 2003). A loop full of the RV enrichment was inoculated on XLD agar. XLD agar relies on the production of hydrogen sulfide, xylose fermentation, and lysine decarboxylation for the differentiation of *Salmonella* from non-pathogenic bacteria. Typical colonies of *Salmonella* appear red with or without black centers, while atypical colonies are yellow with or without black centers. In this research, Salmonella was not detected using the conventional culture method.

The viable bacteria cell count per g or ml of yoke, beef, prawn and pork on XLD agar was calculated to be approximately $8x10^1$, $5.5x10^2$, $1x10^1$ and $3.9x10^2$ cfu respectively. The bacterial cells isolated from beef, yoke and prawn were all gram-negative rods and catalase-positive and are indicative of organisms such as *E. coli, Citrobacter, Klebsiella* and *Proteus* (www.oxoid.com). Gram-positive cocci and weakly catalase-positive bacterial cells were isolated from prawn samples which is indicative of *Enterococcus* spp. Apart from the possibility of *Salmonella* not being present in the food samples due to adequate and effective surveillance measures that have been implemented, several obstacles might have resulted in the inability to detect *Salmonella*. The enrichment cultivation step can fail to produce the desired impact, which is to favour growth of *Salmonella*. This might be as a result of antibacterial components present in the food sample which can lead to the establishment of non-culturable but viable cells of the target organism (Taskila *et al.,* 2012).

As shown by Tatsadjieu *et al.* (2009) certain bacteriocins of lactic acid bacteria also inhibit the growth of *Salmonellla*. Muniesa, *et al.* (2005) indicated in their research 'The effect of bacteriophages on the outcome of *Salmonella* and *Shigella* enrichment culture', that the presence of bacteriophages in food samples produces a significant reduction in *Salmonella*. Though MacCann *et al.* (2005) showed that the inhibitory effect of food components which are capable of producing a false negative result also depends on the enrichment broth and the food sample. It has been identified that, though the enrichment cultivation of *Salmonella* is carried in two steps; pre-enrichment and selective enrichment, there is a possibility that the background microbiota could outgrow the target microorganism during the pre-enrichment stage. For example, the growth of enteric bacteria could be inhibited by the presence of lactic acid bacteria (Vold, *et al.,* 2000). Sveunm and Harman (1997) established the use of a selective supplement in the non-selective culture medium that would favour the growth of *Salmonella*. This was achieved by the gradual release of iodine and selenite into the non-selective medium via a wax-coated gelatine capsule. Jensen *et al.* (2003) in a related study also showed that overgrowth of the background microbiota could be reduced by the addition of antibiotics such as novobiocin and malachite green to the non-selective enrichment cultivation. Though is of contrasting opinion that the addition of antibiotics at the early stage of cultivation especially in the presence of sub-lethally injured *Salmonella* cells could be a source of stress and risk the recovery of such cells (Taskila *et al.,* 2012). Chen *et al.* (2010) also showed that *Salmonella* can be detected directly, after cultivation in non-selective media with the use of certain detection techniques.

The result of the PCR performed in this research was not conclusive enough to carry out a comparative study, as there were no visible amplicons for the reaction. This would have been interpreted as culture being more effective than PCR, but several studies have been carried out that validate the increased specificity, sensitivity, and accuracy of PCR over culture. Table 5.1, shows a compilation of the results from comparative studies that validate the accuracy of PCR over culture for the detection and identification of *Salmonella* in food samples such as poultry, egg, milk, beef, pork, and their products. However, in the study carried out by Whyte *et al.* (2002), PCR was unable to identify *Salmonella* in 7(22%) samples from which the pathogen was isolated via culture. This resulted in the opinion that a combination of both methods would provide more accurate assessment of the prevalence of food-borne pathogens.

The positive control also did not have any visible bands because the primers used were not complementary to any gene sequence present in *S.* Typhimurium. The non-specificity of the primer sequence to the positive control was identified by carrying out a blast similarity search on the NCBI internet site (http://blast.ncbi.nlm.nih.gov/Blast). Several factors were put into consideration to identify why the PCR assay failed to work. This could have been as a result of the following: error in set up, wrong annealing temperature, inadequate template, primer dimmers, error in cycling, error in gel analysis, DNA not clean or contains inhibitors or Secondary structure in template (McPherson *et al.,* 1995). To combat the fore mentioned probable causes of error, the annealing temperature was lowered from 60ºC to 50ºC, all reagents were checked to identify if they were properly added, if the gel was loaded properly and if the program on the thermal cycler was correct. Conventional PCR methods have also advanced with the development of real-time PCR, multiplex PCR and so on, which are more effective in the detection of

Salmonella. Real-time PCR, also known as quantitative real-time not just allows more rapid detection but also quantification of the PCR product. Conventional PCR methods require post-amplification manipulations such as gel-electrophoresis or heterogeneous hybridisation which sometimes results in a false positive result due to contamination (Taskila *et al.*, 2012). In contrast, real-time PCR is carried out in a closed assay format where by amplification is monitored by an increase in fluorescence at every reaction cycle.

It requires no post-PCR modification and therefore reduces technical complexity, the risk of deriving a false positive result and increased robustness (Taskila *et al.*, 2012; Hein *et al.*, 2006). The two most popular fluorescence reporter systems used in real-time PCR are, the DNA binding dye technology and the 5' nuclease assay which use intercalating SYBR® Green and TaqMan® (http://www premierbiosoft.com) respectively as probes for the detection and quantification of *Salmonella* (Cheung *et al.*, 2012; Taskila *et al.*, 2012; Hein *et al.*, 2006). Other systems such as the molecular beacon-based PCR assay and Scorpion probes (http://www.sigmaaldrich.com) are also in use (Cheung *et al.*, 2012; Taskila *et al.*, 2012).

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