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TECHNICAL REPORT ON MOLECULAR DETECTION OF SOME FOODBORNE PATHOGENS IN READY-TO-EAT GRILLED BEEF SAUSAGES FROM SELECTED STREET VENDORS IN ACCRA USING OPTIMIZED REACTION VOLUME AND SINGLE PCR CONDITION

By

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Abstract

Instantaneous procedures that are sensitive for detecting bacteria pathogens in food are lacking and needed. Preventable food borne diseases continue to affect many particularly in developing countries and this has hindered food security in such areas. Improving pathogen detection methods by exploring the most modern techniques and optimizing these techniques to identify multiple organisms within the shortest period of time is desirable. Hence, this study set out to investigate the usefulness of a single PCR condition to amplify the target genes of four different foodborne pathogens. A single thermal cycling protocol was used to identify and confirm the presence of four different bacteria pathogens namely; *Salmonella spp*, *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* in RTE beef sausages. The data obtained from this study suggest that all the above mentioned organisms were present in RTE grilled beef sausages sampled from various parts of Accra**.** Therefore, the following conclusions can be drawn from the present study; the safety of RTE grilled beef sausages analyzed cannot be assured and the use of the same PCR condition is capable of detecting four pathogens in a shorter time.

Key words: PCR, RTE grilled beef sausages, *Salmonella spp*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*

Introduction

The global burden of foodborne diseases has increase rapidly with the estimated number of deaths from 31 major food safety hazards being 420, 000 in 2010 (WHO, 2015). Foodborne pathogens are a major concern to public health in Ghana and the world at large. Tracking foodborne pathogens in the food processing and retailing industry is of utmost importance for identification,

facilitation of outbreak investigation and rapid action in controlling and preventing foodborne disease outbreaks. Also, this is an important aspect of human health care (Xu *et al.*, 2012).

Meat and meat products in general have been implicated as vehicles for the spread of foodborne pathogens (Yousef, 2014). In Ghana, there are about 24 meat processing firms with 12 in the Greater Accra region located in and around Accra and Tema. Fresh pork sausage, smoked pork sausage, fresh beef sausage, liver sausage, white sausage are some of the sausage products produced amongst other meat products like beef, lamb and pork (Teye, 2010). Meat and meat products either produced locally or imported are retailed by cold stores. Fresh meat contains sufficient nutrients that support the growth of microorganisms and therefore makes meat a very perishable product. Many interrelated factors such as holding temperature, atmospheric oxygen (O_2), endogenous enzymes, moisture, light and most importantly, micro-organisms influence the shelf life and freshness of meat (Lee, 2018; Zhou *et al*., 2010; Eie *et al*., 2007) . Minced meat presents an increased surface area exposure that can easily get contaminated during the mincing process (Zhou *et al*., 2010). In the last decades, the number of incidents caused by spoilage and pathogenic bacteria associated with sausage consumption have increased. These microorganisms include Coliforms, *S. aureus, B. cereus, E. coli, Salmonella* spp, *Listeria monocytogenes*, mould and yeast, which were isolated from different sausage products and processing lines (Yousef, 2014; Afshin *et al*., 2011a; Afshin *et al*., 2011b; Güngör & Gökoğlu, 2010).

There are several methods for the detection of these foodborne pathogens such as the culture-based methods, biosensor methods, immunological methods and molecular biology-based methods (Priyanka *et al*., 2016). One simple and rapid molecular biology-based method is the use of conventional Polymerase Chain Reaction (PCR) technique in the detection of low numbers of bacteria pathogens in various food samples including sausages and other Ready-to eat (RTE) foods

using gene specific primers that are able to amplify the target gene of interest to differentiate one organism from the other (Iyer & Kumosani, 2010).

RTE grilled sausage joints have sprung up all over the country with most of the vendors either using locally processed sausages or imported sausages sold by cold stores. Most of these vendors do not observe food safety practices in general which has raised concerns about the microbial safety of these RTE sausages. Therefore, this work was conducted to detect the presence or absence of *Salmonella spp, Escherichia coli, Bacillus cereus* and *Staphylococcus aureus* from RTE grilled beef sausages from selected street vendors in Accra using a simple, rapid and standard PCR method employing same conditions.

Materials and Methods

Sample collection and Study area

A total of twenty-five RTE grilled beef sausage samples were purchased randomly from street vendors located at five different suburbs in the Greater Accra Region into sterile bags. Five samples each was purchase from Madina (M), Ashaiman (AS), Gbawe (G), Teshie (T) and Achimota (A) making up a total of 25 samples. They were aseptically transported under cold chain on ice to the laboratory for analysis.

Extraction of bacteria DNA from RTE grilled beef sausage samples

For each RTE grilled sausage sample, 10 g was weighed, homogenised in 20 ml of Luria–Bertani (LB) broth and incubated at 37 ℃ for 48 h. Then, 1ml of each culture was inoculated into a 10 ml

of freshly prepared Luria–Bertani (LB) broth and incubated at 37 ℃ for 24 h in a shaking incubator at 120 rpm. Subsequently, 1ml of each 24-hour culture was centrifuged at 13000xG for 3 mins (4 ℃). The resulting DNA pellets were purified by washing twice in 500 µl 1x TAE (Tris-Acetic-EDTA, pH 8.0) buffer and centrifuged again at 13000xG for 1 min. The DNA pellets were re-suspended in 200 µl of 0.1M TE buffer, vortexed briefly and DNA was extracted from the pellets by boiling for 8 mins. The extracted bacteria DNA was stored at -20 ℃. The same procedure was used for the positive reference cultures (*Escherichia coli* NCCB100282, *Staphylococcus aureus* NCCB 100294, *Bacillus cereus* NCCB 100292 and *Salmonella spp* 20B-1410) all obtained from Health Protection Agency (HPA) Culture Collection, UK.

Polymerase chain reaction and conditions for thermal cycling

Target genes of the four bacteria species investigated were amplified using the direct Polymerase Chain Reaction (PCR) technique with the extracted DNA as template. PCRs were conducted in 12.5 µL reaction mixtures comprising 1.5 µL of DNA template, 6.75 µL hot start master mix (Containing: Taq polymerase, the DNTPs and the reaction buffer), 0.25µl each of the primer pairs (20 µM) listed in table 1 and 3.75 µL nuclease free water. The negative control was prepared using the hot start master mix with the primer and nuclease free water used as DNA template. Positive bacteria cultures from HPA, UK were used as references. The set of primers used were those that were capable of amplifying the target regions of interest in the organism's DNA, specific to that organism alone as shown in Table 1.

The amplification was conducted using a thermocycler (Applied Biosystems, USA). The PCR thermal cycling protocol was as follows; initial denaturation was at 95 ℃ for 5 seconds, then 40 cycles of denaturation at 94 ℃ for 30 seconds, followed by annealing at 55 ℃ for 1 minute,

extension at 72℃ for 3 minutes. The final extension procedure was at 72 ℃ for 10 minutes. Subsequently, the PCR products were held at 4^0C.

Table 1: Primers used and their target in the amplification of pathogenic bacteria

Primer	Primer sequence （5' to 3'）	Target Gene
Ec3	F: GCG CTG TCG AGT TCT ATC GAG C	fliC
Ec4	R: CAA CGG TGA CTT TAT CGC CAT TCC	
Sa1	F: GCA AGC GTT ATC CGG ATT T	Staph 16S rRNA
Sa2	R: CTT AAT GAT GGC AAC TAA GC	
Bc1	F: GAG TTA GAG AAC GGT ATT TAT GCT GC	Bc group (95%) pc-plc
Bc2	R: CTA CTG CCG CTC CAT GAA TCC	
Sm5	F: GGG TGG GCG GAA AAC TAT TTC	bcfC fimbrial usher protein
Sm6	R: CGG CAC GGC GGA ATA GAG CAC	

F: Forward primer, R: Reverse primer

Agarose gel electrophoresis

The amplified DNA (5 µL per lane) were separated on 1.5% agarose gel containing 3 µL of ethidium bromide (Promega, Madison, USA) in 1X Tris –borate EDTA buffer. The gel was run at 100 V for 45 minutes alongside 1Kb DNA Ladder. The bands on the gel were visualized under UV illumination using the gel imager.

Results

E.coli was detected in some of the RTE grilled beef sausage samples. The fragments produced after amplification with the *E.coli* specific primers were about 347bp. The *E.coli* 0157 primers amplified the gene in 13 samples out of the total 25 samples collected representing 52 % of the total sample population. Four (4) samples from Teshie (T), three (3) from Madina (M) and two (2) each from Gbawe (G), Achimota (A) and Ashaiman (AS) respectively as shown in Figure 1.

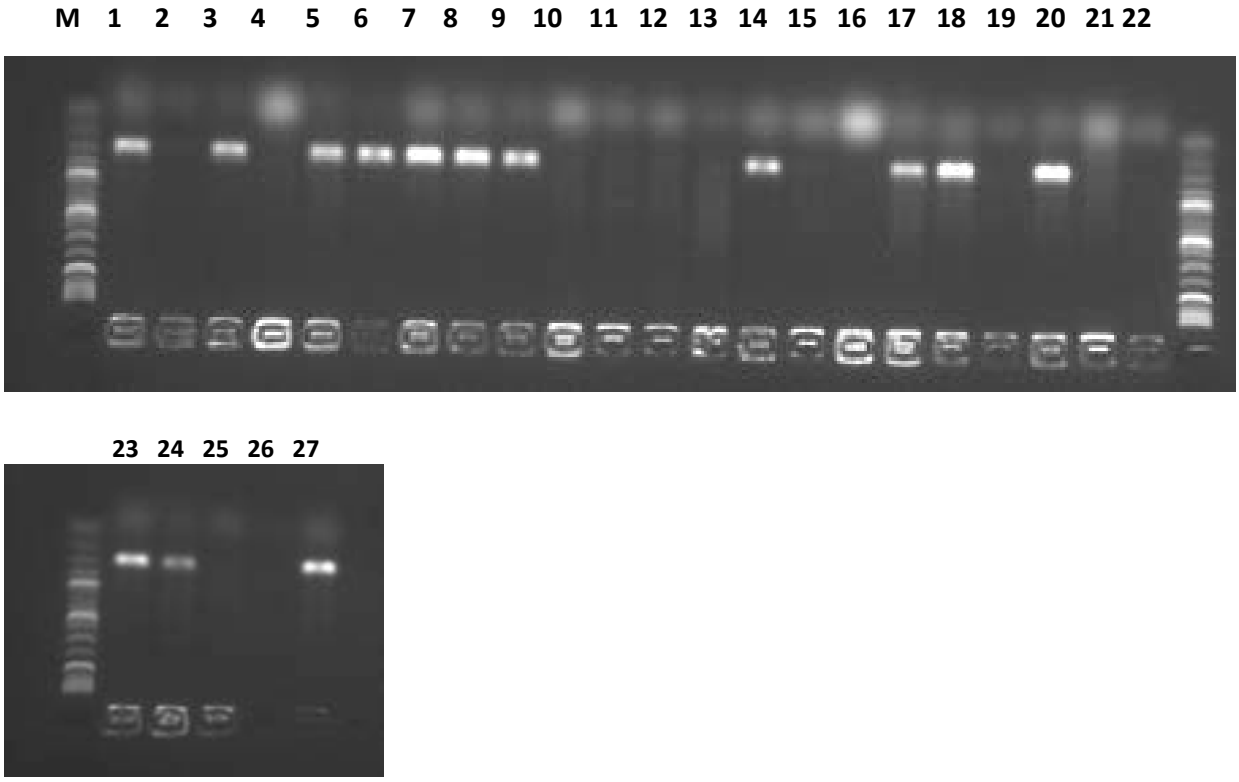

Plate 1: PCR amplification products of *E.coli* 0157 gene obtained from 25 sausage samples using Ec3/Ec4 F/R primer pair. Lanes: 1= AS1, 2=G1, 3=A4, 4=T3, 5=A1, 6=G5, 7=G3, 8=M4, 9=AS3, 10=A3, 11= G4, 12=A5, 13=AS5, 14=M3, 15=M2, 16=AS2, 17=T2, 18=T1, 19=M5, 20=M1, 21=AS4,22=G2; 23=T4, 24=T5, 25=A2, 26= Negative control (product without DNA template) , 27= +Ve = Positive control (347bp) - M= 1kb plus DNA ladder

Figure 1: PCR amplification products of *E.coli* 0157 gene obtained from 25 sausage samples.

Staphylococcus aureus was detected in 22 samples representing 88 % of the total sample population. Therefore *S. aureus* was the predominant pathogen associated with the grilled sausage samples. However, *S. aureus* was not detected in one sample each from Ashaiman (AS2), Madina (M5) and Gbawe (G2) as shown in Figure 2. The size of the fragments produced were in the region of about 597bp.

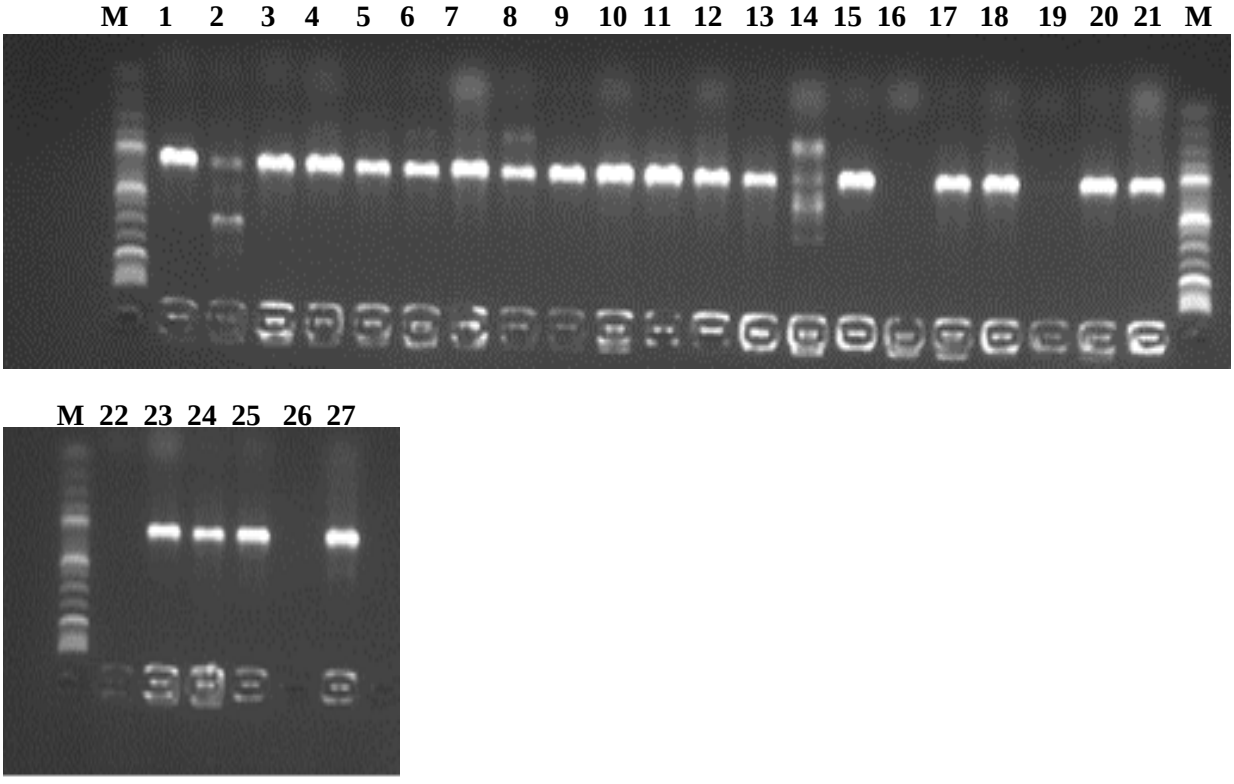

Plate 2: PCR amplification products of *S. aureus* gene obtained from 25 sausage samples using Sa1/Sa2 F/R primer pair. Lanes: 1= AS1, 2=G1, 3=A4, 4=T3, 5=A1, 6=G5, 7=G3, 8=M4, 9=AS3, 10=A3, 11= G4, 12=A5, 13=AS5, 14=M3, 15=M2, 16=AS2, 17=T2, 18=T1, 19=M5, 20=M1, 21=AS4,22=G2; 23=T4, 24=T5, 25=A2, 26= Negative control (sterile nuclease free water) , 27= +Ve = Positive control(597bp) - M= 1kb plus DNA ladder

Figure 2: PCR amplification products of *S. aureus* gene obtained from 25 beef sausage samples.

Bacillus cereus was detected in 11 out of the 25 samples representing 44 % of the total sample population. These were detected in samples from all the five localities as shown in Figure 3. The amplifications showed a band size of about 411bp.

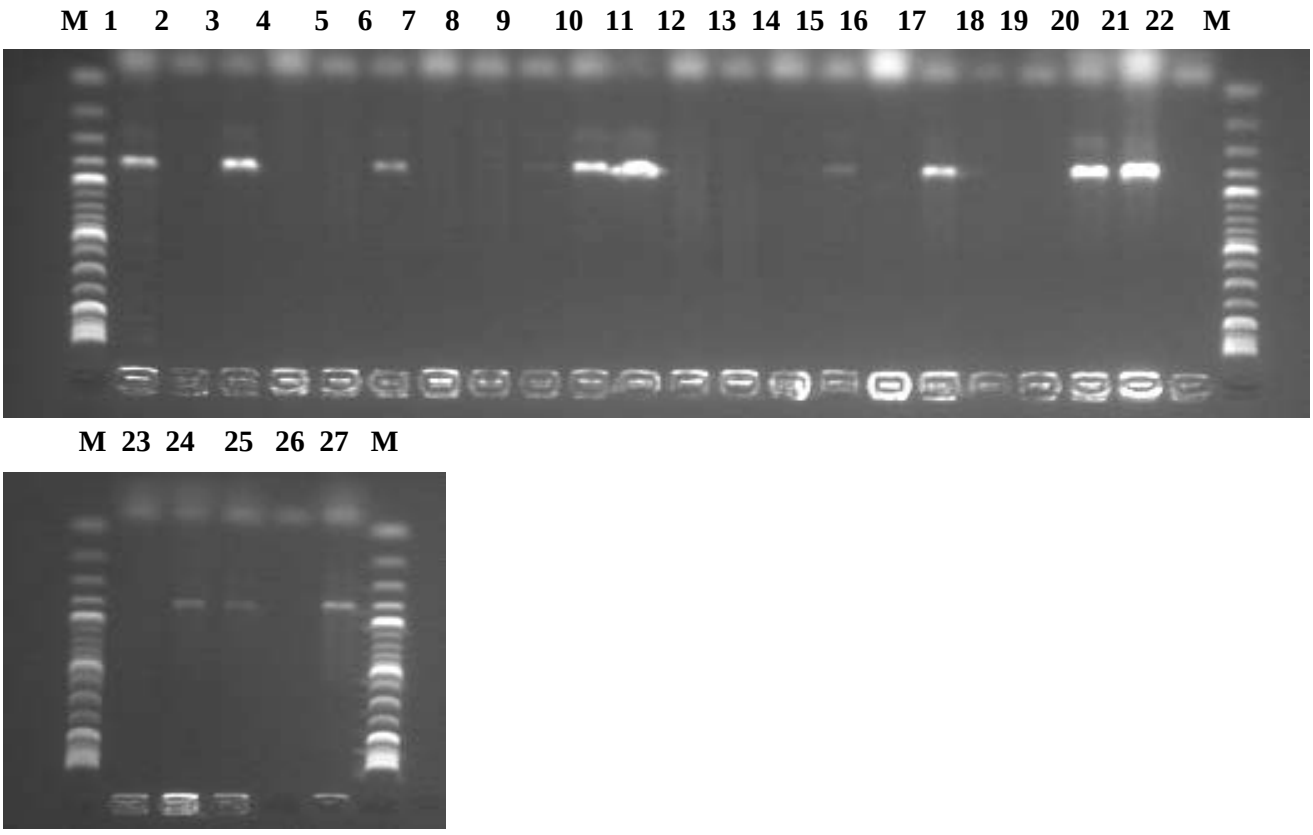

Plate 3: PCR amplification products of *B. cereus* gene obtained from 25 sausage samples using Bc1/Bc2 F/R primer pair. Lanes: 1= AS1, 2=G1, 3=A4, 4=T3, 5=A1, 6=G5, 7=G3, 8=M4, 9=AS3, 10=A3, 11= G4, 12=A5, 13=AS5, 14=M3, 15=M2, 16=AS2, 17=T2, 18=T1, 19=M5, 20=M1, 21=AS4, 22=G2; 23=T4, 24=T5, 25=A2, 26= Negative control (sterile nuclease free water) , 27= +Ve = Positive control(411bp) - M= 1kb plus DNA

Figure 3: PCR amplification products of *Bacillus cereus* gene obtained from 25 sausage samples

Salmonella spp. gene was amplified in the samples in lane 4, 6, 7, 9, 10, 11, 12, 13 and 15 showing faint double and multiple bands as shown in Fig. 4. The fragment sizes varied between 300bp and 400bp. However, only the sample in lane 1 was in line with the reference positive control amplification which produced a fragment of about 400bp indicating differences in the *Salmonella* strains in the samples analysed.

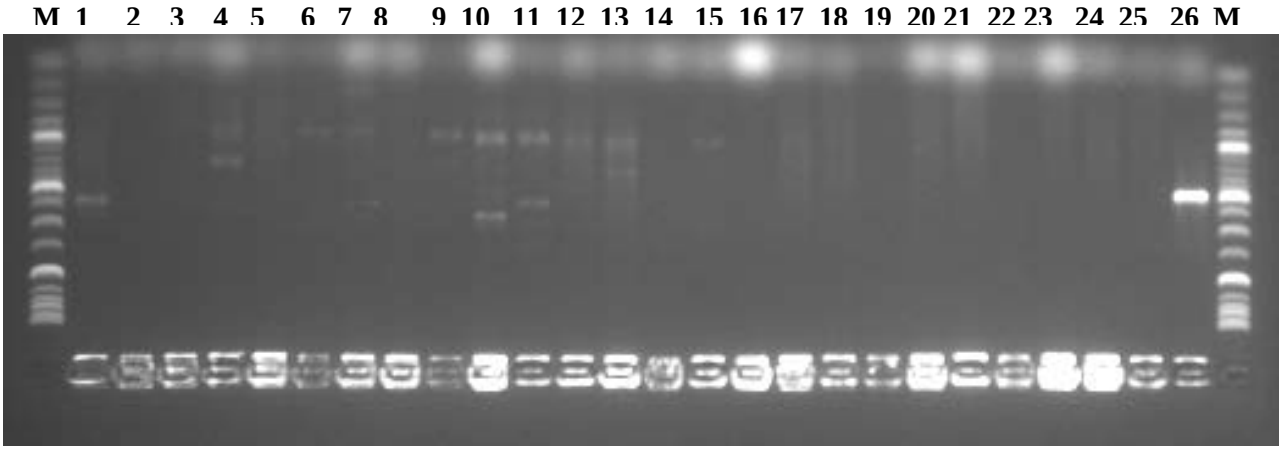

Plate 4: PCR amplification products of *Salmonella spp.* gene obtained from 25 sausage samples using Sm5/Sm6/4 F/R primer pair. Lanes: 1= AS1, 2=G1, 3=A4, 4=T3, 5=A1, 6=G5, 7=G3, 8=M4, 9=AS3, 10=A3, 11= G4, 12=A5, 13=AS5, 14=M3, 15=M2, 16=AS2, 17=T2, 18=T1, 19=M5, 20=M1, 21=AS4,22=G2; 23=T4, 24=T5, 25=A2, 26= Positive control(973bp) - M= 1kb plus DNA ladder

Figure 4: PCR amplification products of *Salmonella spp.* gene obtained from 25 sausage samples

Discussion

A number of factors influence the microbial quality of meat products in general. These include the quality of the raw materials, other additives used during meat processing operations, the effectiveness of the cooking process, sanitation during processing, packaging and handling, maintenance of adequate refrigeration from the processor to the retail level and to the consumer and finally, sanitation during handling at the retail stores (Selvan *et al.*, 2007). Ready-to-eat (RTE) foods must be safe for consumers particularly the vulnerable groups like children, elderly and pregnant women. Therefore the presence of *S. aureus, E.coli, B. cereus, Salmonella* spp in the sampled sausages is of great concern. This clearly indicates that the heat treatment during the grilling process is inadequate and could not eliminate these dangerous pathogens. Most processors purchase the fresh sausages from the cold store during which time it is kept in a freezer. The major issue is how they store it when the sausage is in their possession before grilling. Meat is a rich medium for microbial growth and as such should be kept in a cold storage but this is hardly practised by most sausage vendors who transport meat to the sales points. They are kept at the mercy of the weather, instead of cold conditions for storage. These processors probably believe that the sausage is cooked during processing at the factories and can therefore, be eaten fresh and so does not require a lot of grilling to get rid of potential pathogens. The poor hygienic practices of these vendors and the over exposure to dirty environment may also be another contributory factor. Their display glass cases sometimes have flies trapped in them which can result in the proliferation of microorganisms and the spread of food-borne pathogens. This finding is similar to that of Waskar (2006), who reported that *S. aureus, E.coli*, *Aeromononas* and *Salmonella* spp were found to be the predominant pathogenic bacteria on processed meat samples including sausages and reiterated the need for implementation of stringent quality control checks at all points of

production, processing, distribution and marketing. Similarly, a study revealed that careful handling of ready-to-eat cocktail sausage and maintenance of adequate chill temperature during storage would improve the microbiological quality and enhance the shelf life (Afshin *et al*., 2011a).

Also, the results of this study confirm the reliability of standard PCR technique for the rapid screening of food samples for pathogens. The use of the same PCR condition is capable of detecting four pathogens in a shorter time. Approximately, 30-45 mins for DNA extraction, 4 h for PCR amplification, 2 h for electrophoretic separation and 10 mins for interpretation**.** In addition, the cost of the method for simultaneous identification of food-borne pathogens is expected to be cheaper than the conventional microbiological detection method for each of the four pathogens.

Conclusion

An enrichment stage at a temperature of 37 °C followed by DNA isolation, PCR, gel electrophoresis and visualization is a suitable method for the detection of pathogens in food samples. In addition, the fact that one thermal cycle run can be used to detect more than one pathogen is an added advantage. The safety of RTE grilled beef sausages analysed in this study cannot be assured.

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