INTERNATIONAL JOURNAL OF
POULTRY SCIENCE

CrossMark
← click for updates

Improved Detection of *Campylobacter jejuni*, Listeria *monocytogenes* and *Salmonella* Typhimurium from Raw Meat Products Using Conventional and Newly Developed TaqMan Assays

Giang Nguyen[1], Magda Ismail Abo-Samaha[2,3], Gopal Reddy[2], Mohammed Abdulrahman[2], Temesgen Samuel[2], Abiodun Adesiyun[4] and Woubit Abdela[2]
[1]National Institute for Food Control, Ha Noi, Viet Nam,
[2]Department of Pathobiology, College of Veterinary Medicine, Nursing and Allied Health,
Tuskegee University, Tuskegee, Alabama-36088, USA
[3]Department of Animal Husbandry and Animal Wealth Development,
Faculty of Veterinary Medicine, Alexandria University, Alexandria, Egypt
[4]Department of Production Animal Studies, Faculty of Veterinary Science,
University of Pretoria, Onderstepoort, Pretoria, South Africa

Abstract: The most frequent bacteria contaminating raw meat samples from local retail outlets were determined. Comparison was made between conventional culture method (BIOLOG) and newly developed PCR assays performed on bacterial isolates (PCR-C), directly on enriched broths (Direct PCR-E) or on DNA extracted from enriched broths (PCR-E) of *Campylobacter jejuni*, Listeria monocytogenes and *Salmonella* Typhimurium. Among 150 meat samples (chicken, pork, turkey and beef) tested, Campylobacter, Proteus, Listeria and *Salmonella* were detected in 27.0, 25.0, 19.0 and 3.3%, respectively, by using BIOLOG. Among 150 samples analyzed, the frequency of detection for *C. jejuni*, *L. monocytogenes* and *S.* Typhimurium were 8.0, 2.7 and 2.0% by both the BIOLOG and PCR-C assays. PCR-C and BIOLOG detected the same number of positive samples, 12 for *C. jejuni*, 3 for *S.* Typhimurium, however from the total 4 *L. monocytogenes*, only 3 were isolated correctly by BIOLOG. Both Direct PCR-E and PCR-E detected 13, 2 and 1 positive samples for *C. jejuni*, *S.* Typhimurium and *L. monocytogenes*, respectively. TaqMan PCR assay on enriched broths produced the most rapid and inhibition-free results compared with Direct PCR-E and PCR-C. The PCR-based methods provided results more rapidly (1-2 days versus 6-7 days) and therefore are recommended for improved detection of these pathogens.

Key words: *Salmonella*, *Campylobacter*, *Listeria*, meat, isolation, TaqMan assay

INTRODUCTION
Food borne diseases associated with *Campylobacter jejuni*, *Listeria monocytogenes* and *Salmonella* Typhimurium are among the most prevalent food borne pathogens (Cook *et al.*, 2012). Illnesses associated with these three bacteria account for more than 50% of the mortality from food borne disease (CDC, 2010). Despite the improved quarantine and other control and preventive measures, new outbreaks associated with these pathogens continue to occur, with high mortality and marked economic loss (Karch *et al.*, 2012).
Campylobacter jejuni is the most recognized species involved in 95% of campylobacter outbreaks and sporadic illnesses (Adedayo and Kirkpatric, 2008). It is considered the leading cause of bacterial gastroenteritis in the developed world, thought to infect 3 million people a year in the US alone, at a cost to the economy exceeding $4 billion (Wilson *et al.*, 2008).
Human listeriosis is usually associated with ready-to-eat meat (Gallagher *et al.*, 2013). Illnesses associated

with *L. monocytogenes* have a higher mortality rate than any other food borne illnesses. This pathogen is responsible for annual deaths of more than 250 people in the U.S., particularly among the elderly, fetuses in utero and the immuno-compromised (Scallan *et al.*, 2011). The United States Department of Agriculture (USDA) identified deli meats, red meat, poultry and egg as the most important sources of infection by this pathogen (FSIS, 2009).
Salmonella Typhimurium is among the most common *Salmonella* serovars causing infections in the US (CDC, 2011).
Meat and meat products from retail outlets have been documented to be contaminated at different frequencies and implicated in infections and outbreaks associated with *C. jejuni* (Cook *et al.*, 2012; Rokosz *et al.*, 2014), *L. monocytogenes* (Bohaychuk *et al.*, 2006; Lamden *et al.*, 2013) and *S.* Typhimurium (CDC, 2014; Phillips *et al.*, 2008).

Corresponding Author: Woubit Abdela, Department of Pathobiology, College of Veterinary Medicine, Nursing and Allied Health, Tuskegee University, Tuskegee, Alabama-36088, USA

Microbial analysis of foods is an integral part of management of microbial safety in the food chain. Conventional methods for the isolation of *C. jejuni, L. monocytogenes* and *S.* Typhimurium are primarily based on bacteriological, biochemical and serological assays. These procedures are labor-intensive, expensive, complicated and time-consuming in many instances requiring from 5 to 10 days to be completed (Kim and Cho, 2008; Sheare *et al.*, 2001). Real-time PCR assays for food microbiology have been developed into commercial products and are generally highly automated and minimize the number of operations involved, reducing the risk of contamination and rapidly and accurately detecting bacterial pathogens in foods (Cremonesi *et al.*, 2014; Kawasaki *et al.*, 2010; Suo *et al.*, 2010).

To date, a number of simultaneous-detection assays have been developed for these pathogens in combination with other organisms (Lee *et al.*, 2014; Shu *et al.*, 2014; Suo *et al.*, 2013). Furthermore, the sensitivity and specificity of a number of these tests have been determined using experimentally inoculated meat and meat products and other foods (Fakruddin *et al.*, 2013b; Suo *et al.*, 2010). However, there is a dearth of information on these developed methods being validated in a wide array of naturally contaminated meat and ready-to-eat meat products collected from retail supermarkets.

In this study, we used combined traditional culture and PCR methods to detect the presence of three food borne pathogens (*C. jejuni, S.* Typhimurium and *L. monocytogenes*) in meat samples from retail supermarkets. We also investigated the possible use of multiplexed conventional and a TaqMan PCR-based assays for simultaneous and rapid identification of the three food borne pathogens and compared the recovery of these pathogens between the PCR assays and the traditional methods.

MATERIALS AND METHODS

Type of samples: A total of 150 samples of raw meat (Pork, Beef, Turkey and Chicken) were randomly collected during three different visits to three different retail stores in Tuskegee, Alabama between September 07 and December 30, 2013. The samples collected comprised the following: 63 chicken samples (neck-3, leg-15, gizzard-15, wing-12, thigh-12, breast-3 and back-3), 45 pork samples (sausage-18, meat-15 and hamburger-12), 33 turkey samples (ground turkey-18, leg-6, neck-6 and wing-3) and 9 ground beef samples.

Selection of pathogens: For this investigation, the primary bacterial pathogens of interest were *C. jejuni, L. monocytogenes* and *S.* Typhimurium because of their reported implications in meat-borne infections and diseases (Adedayo and Kirkpatric, 2008; Almeida *et al.*, 2010; Bohaychuk *et al.*, 2006; CDC, 2010; Cook *et al.*,

2012; Fakruddin *et al.*, 2013a). Nevertheless, other pathogens present in the different meat samples were also cultured during the study.

Preparation of samples and culture for bacterial pathogens: Isolation and culturing of *Campylobacter* were always conducted with the AnaeroPak system (BD Diagnostics, Spark, Maryland, USA) under microaerophilic conditions created by using a 10% CO_2, 10% H_2, 80% N_2 gas mixture and Campy pack (Remel Inc., Lanexa, Kansas, USA). The FDA procedure was followed with slight modification (Hunt *et al.*, 2013). A 25 ml portion of a meat sample rinse in Buffered Peptone Water (BPW) was centrifuged at 3000 x g at 22°C for 3 min. Twenty-milliliters of the supernatant was discarded and 5 ml of the sediment vortexed for 30 s and mixed with 45 ml Bolton broth (Remel Inc., Lenexa, Kansas, USA). The broth cultures were first incubated at 37°C for 4 h and then at 42°C for 44 h. One ml of the 48 h enrichment culture was aseptically poured into 1.5 ml micro centrifuge tube (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and centrifuged at 17500g for 10 min at 4°C. The supernatant was carefully discarded and the pellet was streaked on Campylobacter Blood-Free Selective Agar Base plates, containing Campylobacter charcoal differential agar (CCDA) selective supplements (Remel Inc., Lenexa, Kansas, USA). Streaked plates were incubated under micro-aerophilic conditions at 42°C, for 48 h after which the plates were examined for colonies with characteristic phenotypic appearance of *Campylobacter* which were small, gray and drop-like or small and shiny or slimy. Presumptive *Campylobacter* colonies were subcultured on blood agar plates and incubated under microaerophilic conditions for 48 h at 42°C. Single colonies (3-5) on blood agar plates were selected for Gram staining followed by oxidase, catalase and hippurate tests (Remel Inc., Lanexa, Kansas, USA). Identification to the species level was achieved using BIOLOG GNIII Microbial Identification System (Biolog, Hayward, California, USA).

For the isolation of *Listeria* 25±0.5 g of meat samples were placed into a sterile Whirl-Pak bag (Fisher Scientific, Pittsburg, Pennsylvania, USA) and mixed with 225±5 ml of ONE Broth-Listeria selective enrichment broth (Remel Inc., Lanexa, Kansas, USA), thoroughly shaken and incubated at 37°C for 24 h. A loopful of the suspension was inoculated onto Brilliance Listeria agar (Remel Inc., Lanexa, Kansas, USA) and 1 ml was used to inoculate fresh 10 ml of ONE Broth-Listeria selective enrichment broth (OBLSEB) and was incubated at 37°C for 24 h. Following incubation, a loopful from the 10 ml broth suspension was streaked onto Brilliant-Listeria agar and incubated aerobically at 37°C for 24 h. Isolated colonies resembling *Listeria* were sub-cultured onto Brain Heart Infusion Agar (BD Diagnostics, Spark, Maryland, USA) with 5% sheep blood (Remel Inc.,

Lanexa, Kansas, USA) and incubated at 37°C for 24 h before using the BIOLOG GNIII System (Biolog, Hayward, California, USA) for further identification of *Listeria species*.

In the case of *Salmonella* 25±0.5 g of meat samples were placed into a sterile Whirl-Pak bag (Fisher Scientific, Pittsburg, PA) and mixed with 225±5 ml of BPW (BD Diagnostics, Spark, MD, USA). The bags were shaken thoroughly and incubated at 37°C for 20-24 h. After incubation, 0.5 and 0.1 ml of the homogenous suspension was transferred into 10 ml Tetrathionate broth (Remel Inc., Lanexa, Kansas, USA) and 10 ml Rappaport-Vassilliadis broth (Remel Inc., Lanexa, Kansas, USA), respectively and the broth cultures were incubated at 42°C for 20-24 h for selective enrichment. Thereafter, a loopful of each broth culture was sub-cultured onto XLT4 (Xylose Lysine Tergitol 4) agar (Remel Inc., Lanexa, Kansas, USA) and Brilliant Green Sulfa (BGS) agar (Remel Inc., Lanexa, Kansas, USA) and incubated at 37°C for 20-24 h. Suspect colonies showing phenotypic characteristics of *Salmonella* were picked from each plate and inoculated onto blood agar plates to obtain pure cultures before performing biochemical tests for identification of *Salmonella* spp. using standard methods API 20E and BIOLOG GNIII (Biolog, Hayward, California, USA). Serotyping of the *Salmonella* isolates was kindly performed by the Wisconsin State Veterinary Diagnostic Laboratory, Madison, Wisconsin.

Strategy used for the detection of the three organisms by PCR: Figure 1 depicts the three different PCR steps used to detect the three main pathogens: 1 = Direct PCR-E (PCR directly from enrichment broths), 2 = PCR-E (PCR using DNA extracted from 1ml enrichment broth followed by concentration by centrifugation) and 3 = PCR-C (PCR from DNA extracted from suspected colony aseptically picked and re-suspended in trypic soy broth (Remel Inc., Lanexa, Kansas, USA). All samples were subjected to conventional PCR assay and those DNA samples that tested positive were further analyzed using TaqMan real time PCR assays. PCR-C was run in all the suspected colonies regardless of the BIOLOG results. Control type cultures (Table 1) and genomic DNA from each of the three organisms were purchased from ATCC (Manassas, Virginia, USA).

DNA was extracted from 1 ml each of Salmonella, Campylobacter and Listeria enrichment broths collected after complete homogenization and from suspected colonies re-suspended in 1 ml of tryptic soy broth. Briefly, enrichment broths were vortexed for complete homogenization and centrifuged at 8,900 g for 10 min. Supernatants were discarded and the pellets were re-suspended in 200 µl of PrepMan® Ultra Sample Preparation kit and DNA was extracted by boiling as recommended by the manufacturer (Applied Biosystems, Foster City, California, USA). The quality of

Table 1: List of ATCC cultures used in the study

Species/strains	Origin
Genus *Salmonella*	
Salmonella Typhimurium LT2 ATCC® (700720D-5™)	ATCC
Salmonella Typhimurium ATCC®51812™	ATCC
Salmonella Typhimurium strain ATCC®700730™	ATCC
Salmonella Typhimurium strain MZ1589 ATCC®BAA-1836™	ATCC
Genus *Listeria*	
Listeria monocytogenes ATCC (®11456a™)	ATCC
Listeria monocytogenes ATCC (®11060™)	ATCC
Genus *Campylobacter*	
Campylobacter jejuni subsp. *jejuni* (ATCC® 33560™)	ATCC
Campylobacter jejuni subsp. *jejuni* ATCC 42834	ATCC
Campylobacter jejuni subsp. *jejuni* (ATCC® 33292™)	ATCC
Campylobacter jejuni subsp. *jejuni* ATCC® 29428™	ATCC
Campylobacter jejuni subsp. *jejuni* ATCC® 33291™	ATCC
Campylobacter coli ATCC 47833	ATCC

all the DNA specimens was assessed spectrophotometrically using Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, DE). DNA from enrichment broth of the three organisms was tested to verify the identification without the routine culturing procedure.

Design of primers and probes: Completed genome sequence data of all of *Listeria monocytogenes* and *Campylobacter jejuni subsp. jejuni* were retrieved from the NCBI (http://www.ncbi.nlm.nih.gov/genomes/lproks. cgi) microbial genome database. Highly specific primers LM-R/F and CJ-F/R were designed using V-NTI Advanced-11 (Invitrogen, USA). These primers were examined for specificity using BLAST (http://www. ncbi.nlm.nih.gov/blast/) then further analyzed in-silico (http://insilico.ehu.es/PCR/) for specific binding across the genome sequence of all strains under similar species. Primers (STM2 F/R) for specific identification of *Salmonella* Typhimurium were adopted from Woubit *et al.* (2012). Primers were designed in such a way that they could also be used for conventional PCR by assigning different gene fragment sizes for easy gel electrophoresis analysis. Internal TaqMan probes ROX-CJ-probe, Cy5-LM-probe and FAM-STM-probe were designed using IDT-PrimerQuest™ Tool, containing Iowa Black quencher dye at 3' end. All the three primer pairs and probes (Table 2) were synthesized and ordered from Integrated DNA technologies (IDT, Coralville, IA).

Primers for *C. jejuni* and *L. monocytogenes* and probes for specific and multiplex amplification of the three organisms were successfully designed from genes encoding for cdtA cytolethal distending toxin subunit A and mpl Zinc metalloproteinase respectively. Primer previously designed and validated from putative DNA repair ATPase for specific identification of *S.* Typhimurium (Woubit *et al.*, 2012) was used. They were designed to provide simultaneous identification of the three organisms generating PCR product lengths in the
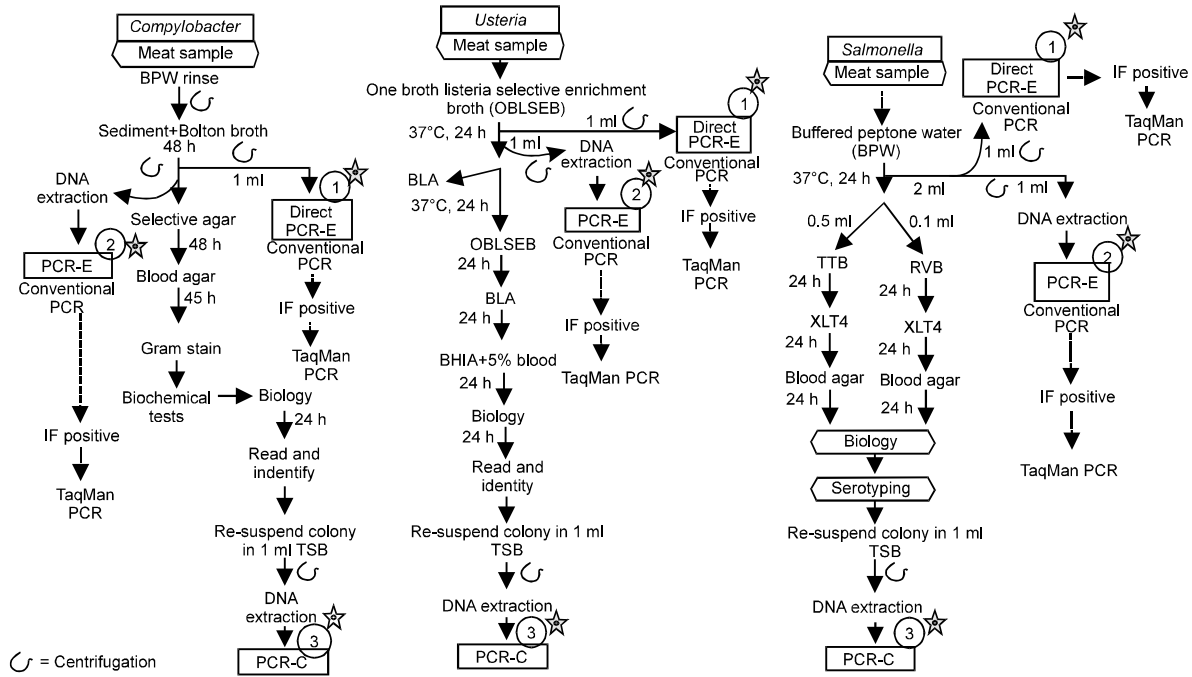
Fig. 1: Flow-chart used for the isolation and identification of the three bacterial pathogens from different food samples using conventional culturing method followed by concurrent Biolog and conventional PCR. The broken arrows indicate PCR performed directly from enrichment and are identified by number 1 and a star sign (Direct PCR-E). PCR from DNA extracted enrichment broth is indicated by number 2 and a star sign (PCR-E). The last PCR-C was performed on DNA extracted from a colony re-suspended in 1 ml tryptic soy broth, indicated by number 3 and a star sign. All steps shown with the solid arrow are concurrently performed

Table 2: List of primers and probes used in the study

Name	Size	5'-3 Sequence	Origin
Campylobacter jejuni subsp. *Jejuni*			
CJ-probe	24	/56ROXN/CAATCCTCGCTTTAAAGACGAAGC/3IABkFQ/	This study
CJ-R	21	CAATAGTGCCAACAATAATGC	This study
CJ-F	20	ATTTGAACCGCTGTATTGCT	This study
Listeria monocytogenes			
LM-Probe	27	/5Cy5/ACTTTTCACATGAGCTTCACTCTGACC/3IABkFQ/	This study
LM-R	21	AACAATCCAACTCGCAACTTC	This study
LM-F	20	AATCAAGGTCCACGTCACAC	This study
Salmonella Typhimurium			
STM-Probe	23	/56-FAM/CCACAACCACCGCTTCTTTGCGC/3IABkFQ/	This study
STM2-F-M	20	CGTTCACCCATGGCTAACTG	Woubit *et al.* (2012)
STM2-R-M	24	GACATTCTAGCTAACCAGCTTGCT	Woubit *et al.* (2012)

order of 384 bp, 274 bp and 189 bp for *C. jejuni* and *L. monocytogenes* and *S.* Typhimurium, respectively. Reporter dyes FAM, ROX and Cy5 with emission wavelength of 520, 602 and 667 were used for simultaneous TaqMan amplification of *S.* Typhimurium, *C. jejuni* and *L. monocytogenes*, respectively. The optimal probe to primer concentration ratio was found to be 800 nM to 900 nM for TaqMan assay with IAC at final concentration of 5 ng in 20 μl PCR reaction volume.

PCR-optimizations: PCR was set up in an isolated PCR station (AirClean Systems, NC) that was UV-sanitized daily and after each use. Master mixes for conventional and TaqMan PCR were optimized following the manufacturers' instructions. Primer concentrations of 150, 200, 300, 600 and 900 nM were tested using 30 ng of genomic DNA of *C. jejuni* ATCC® 33560™, *Listeria monocytogenes* Li 2-ATCC®19115D-5™ and *S.* Typhimurium LT2 ATCC® (700720D-5™). Assays were performed separately in triplicates for each of the pathogen. The optimum primer concentration was used to determine the optimum probe concentrations for the TaqMan-based assay in the range 200-800 nM, with increments of 100 nM. Brilliant III Ultra-Fast QPCR

master mix (Agilent Technologies, Santa Clara, CA) was used to perform the PCR after primer and probe optimization.

Conventional PCR: Conventional PCR was performed in 20 µl final volume containing 0.2 µM of forward and reverse primers, 10 µl of Pwo Master mix containing 1.25 U of Pwo enzyme, 2 mM $MgCl_2$ and 0.2 mM dNTPs (Roche Diagnostics, Mannheim, Germany). The same reaction mix was used for simultaneous assay by reducing the amount of H_2O to make up the final volume. The PCR amplification program for this initial assay consisted of 2 min at 95°C, followed by 30 cycles of 15 seconds at 95°C, 15 sec at 61°C and 15 sec at 72°C using Master cycler pro (Eppendorf, Hamburg, Germany). Presence of single bands was analyzed using gel electrophoresis.

Real-time PCR selectivity: As defined by the MicroVal Protocol/ISO (International Organization for standardization) 16140:2003, selectivity which is a measure of the degree of non-interference in the presence of non-target analytes, was evaluated in terms of inclusivity, exclusivity and by the inclusion of TaqMan exogenous internal amplification control (IAC) (Applied Biosystems, Foster City, CA) to confirm presence of the desired target and absence of PCR inhibitor in our TaqMan assay. The IAC was detected using VIC-labeled probe.

Sensitivity and specificity assay of real-time PCR: Approximately 10^8 cfu/ml of each of the three organisms was serially diluted 10-fold up to 10^{-7} and plated on tryptic soy agar after which the CFU per ml of cell suspension was determined for each of the microorganisms. A pool of the three organisms was then prepared by mixing 333 µl of from each dilution of the three organisms in the same tube and vortexed before subjecting the mixture to DNA extraction for PCR analysis.
Specificity was determined by running each of the specific primers for all the bacterial isolates, enrichment broths and those obtained from ATCC (Table 1) in the present study.

Validation of PCR: The three specific primers were initially validated with standard culture collections representing the three-food borne pathogens (Table 1). Furthermore, each specific primer was validated using a total of 113 isolates obtained in this study using protocol described in Fig. 1.

Detection of *C. jejuni, L. monocytogenes* and *S. Typhimurium* by conventional and TaqMan multiplex PCR assays: All samples that tested positive for *C. jejuni, L. monocytogenes* and *S.* Typhimurium by both BIOLOG and conventional PCR which were conducted concurrently were thereafter subjected to TaqMan multiplex PCR assays.

RESULTS

Isolation and identification of bacteria: Using the conventional culture methods followed by identification with the BIOLOG GN III system, of a total of 150 samples comprising 15 different parts of raw meat, 40 (27.0%), 37 (25.0%), 28 (19.0%) and 5 (3.3%) were contaminated with microorganisms belonging to the genera *Campylobacter, Proteus, Listeria* and *Salmonella*, respectively (Fig. 2). Amongst chicken samples, chicken legs were contaminated with a total of 7 bacterial species (*P. mirabilis, P. penneri, C. jejuni*, G. palaticanis, *L. monocytogenes*, unidentified bacterium and *L. innocua*); among pork samples, pork sausages were contaminated with 4 bacterial species, (Proteus spp., *S.* Infantis, *L. innocua* and *L. welshimeri*); among turkey samples, turkey neck had the highest variety of contaminants, 3 species (Proteus spp., *S.* Reading and *C. jejuni*), while among beef samples, two species of Listeria (*L. ivanovii* ss *londoniensis* and *L. innocua*) were isolated from ground beef.

Detection of *C. jejuni, L. monocytogenes* and *S. Typhimurium* in chickens by multiplex PCR assays: Figure 3 shows the data from both multiplex assay systems (conventional and TaqMan) performed on the three pathogens tested in the current study. Multiplex PCR using either the conventional or TaqMan assays provided successful amplification of the three organisms in one tube. In the conventional PCR, DNA fragments from the three organisms were clearly separated in 1.5% agarose, with the product sizes of 384, 274 and 189 bp for *C. jejuni, L. monocytogenes* and *S.* Typhimurium, respectively. Similarly, validation of the multiplex TaqMan assays from cultures of these organisms identified them based on the three reporter dyes, ROX for *C. jejuni*, CY5 for *L. monocytogenes* and FAM for *S.* Typhimurium. Simultaneous amplification of the IAC in the TaqMan assays revealed the absence of any PCR inhibition.

Sensitivity and specificity of PCR assays: The simultaneous sensitivity assay provided a lower detection limit of approximately 44 cfu/ml for *C. jejuni*, 105 cfu/ml for *L. monocytogenes* and 40 cfu/ml for *S.* Typhimurium. In all the PCR reactions, the IAC was successfully amplified at a final concentration of 5 ng in 20 µl.
Specificity was analyzed by testing non-target organisms using the newly developed primers and the assay was 100% specific as none of the non-targets were amplified either with the conventional or the TaqMan assays.

Validation of PCR assays: The validation assay performed for the three primers on all the 113 isolates
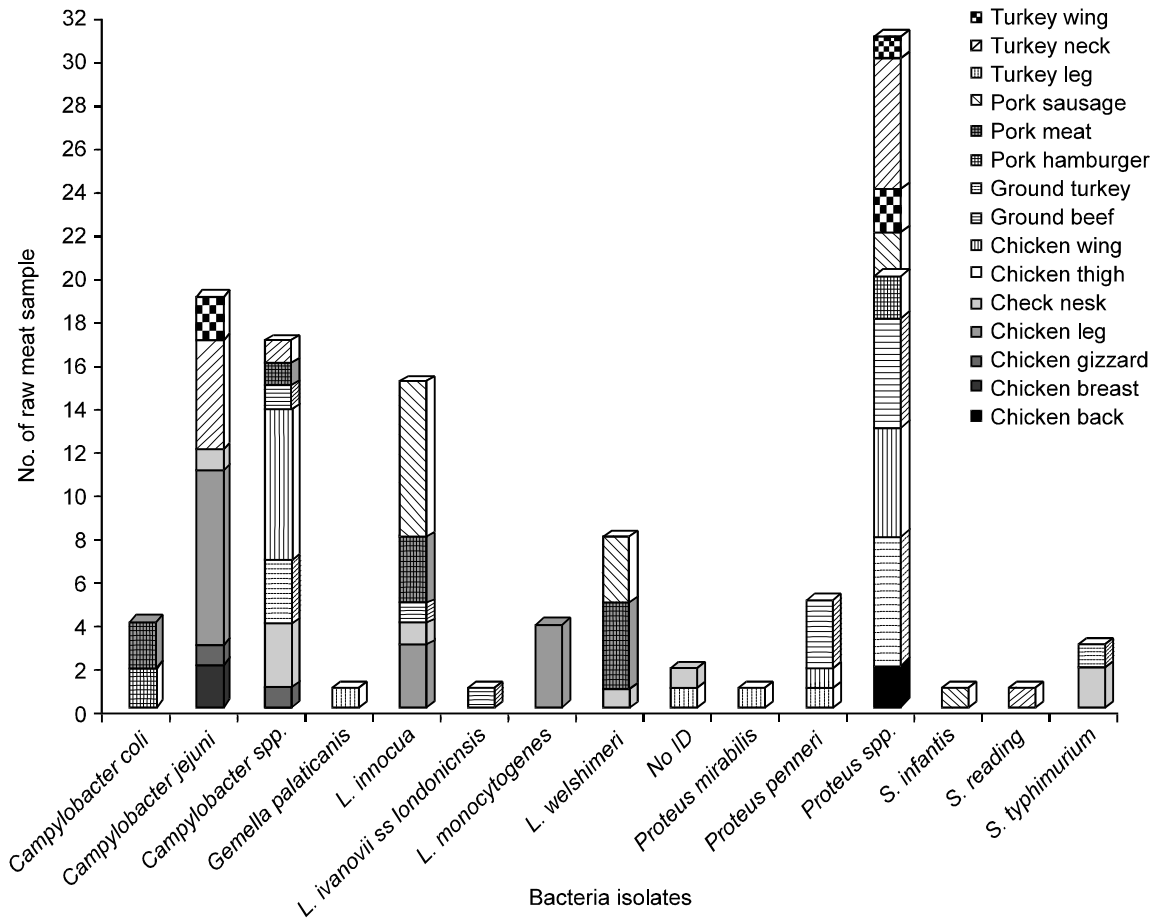
Fig. 2: Types of bacteria isolated from raw meat samples purchased from retail outlets by the conventional culture
methods

by the protocol described in Fig. 1 and the ATCC cultures
(Table 1) provided specific amplification of the target
organisms. The assay was determined to be specific in
both conventional and TaqMan assays.

Comparison of three PCR assays for detection of
C. jejuni: Figure 4 displays data of TaqMan PCR assays
for *C. jejuni* following three different PCR procedures
described in Fig. 1 (Direct PCR-E, PCR-E, PCR-C); Early
results with low Ct (cycle threshold) values of 11-17
were obtained in PCR-E, when DNA was extracted from
enriched broth by centrifugation prior to PCR. It was of
interest to detect that this approach appeared most
efficient in providing the earliest detection of *C. jejuni*
from chicken breast and leg samples compared to
Direct PCR-E and PCR-C methods. Furthermore, the
relatively lower Ct value for the IAC and *C. jejuni*
amplified from PCR-E demonstrated the least PCR
inhibition associated with this approach as compared
with the assays in Direct PCR-E and PCR-C.

Comparison of rates of pathogen isolation by the
BIOLOG system and PCR on enrichment broth
cultures: A comparison of the isolation and detection

rates for the three pathogens by the BIOLOG, PCR
performed on enrichment broths (Direct PCR-E and
PCR-E) and PCR done on bacterial culture (PCR-C) is
shown in Fig. 5. For both *C. jejuni* and *S.* Typhimurium,
of the 150 samples examined, the same number, 12
(8.0%) and 3 (2.0%) respectively were found to be
positive by both PCR on culture and by BIOLOG
methods. However with *L. monocytogenes*, PCR
performed on culture yielded 4 (2.7%) positive samples
compared with 3 (2.0%) samples by the BIOLOG
method. One of the isolates was identified by BIOLOG
as *L. ivanovii*, which resulted in the discrepancy
between the two methods. PCR performed on enriched
samples identified 13 positive compared with PCR
performed on colonies or the BIOLOG method (12 each)
while the reverse was the case with *L. monocytogenes*
and *S.* Typhimurium. The overall small number of
samples positive for *L. monocytogenes* or *S.*
Typhimurium in this study makes it difficult to make
meaningful comparisons.
Figure 6 displays the frequency of detection of the three
pathogens of interest by PCR-C, Direct PCR-E and PCR-
E. The overall frequency of detection of *C. jejuni, L.*
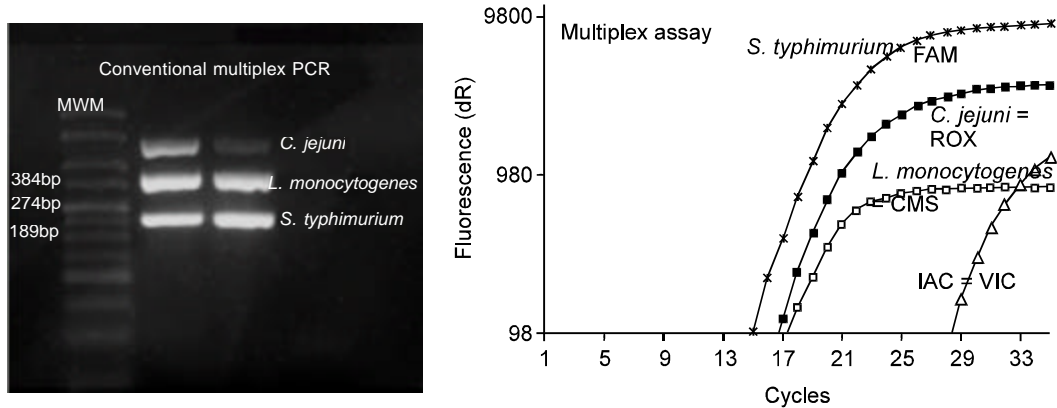*monocytogenes* and *S.* Typhimurium from the 150

Fig. 3: Conventional and TaqMan multiplex PCR amplification of the three food-borne pathogens

samples processed was 17 (11.3%), 4 (2.7%) and 3 (2.0%), respectively, using PCR from cultures and enrichment broths. Chicken legs yielded the highest number of samples positive for *C. jejuni*, (8/15) and *L. monocytogenes*, (3/15) and *S.* Typhimurium (2/15) were positive for *S.* Typhimurium.

Agreement (both tests yielding same results either positive or negative on the same sample) between PCR-E (Direct PCR-E, PCR-E) and PCR-C (conventional), or between PCR-E and TaqMan PCR were the same, 47.1% (8 of 17), 25.0% (1 of 4) and 67.0% (2 of 3) for *C. jejuni*, *L. monocytogenes* and *S.* Typhimurium, respectively (Fig. 5). Also, the frequency of both PCR-E positive but PCR-C negative was 0.0% for both *L. monocytogenes* and *S.* Typhimurium but 29.4% (5 of 17) for *C. jejuni*. Again, the frequency of PCR-E negative but PCR-C positive was high for *L. monocytogenes*, 75.0% (3 of 4) compared with 20.0% (1 of 5) and 23.5% (4 of 17) for *S.* Typhimurium and *C. jejuni*, respectively.

DISCUSSION

The three genera of bacteria (*Campylobacter, Listeria* and *Salmonella*) targeted in the current study have been associated with food borne infections and diseases as reported by others (Mercado *et al.*, 2012; Much *et al.*, 2007). Although *C. jejuni*, *L. monocytogenes* and *S.* Typhimurium are predominantly implicated in meat-borne epidemics (Mercado *et al.*, 2012; Raguenaud *et al.*, 2012), *Proteus spp.* recovered from the meat samples studied are frequently implicated in food spoilage (Lucia *et al.*, 1993). The frequency of isolation of 27.0% found for Campylobacter spp. in the variety of meat samples studied is lower than published reports which used similar methods, where 47.5% (Sison *et al.*, 2014) and 61.2% (Sammarco *et al.*, 2010) of the meat samples were positive for the organism. It was no surprise that chicken samples were contaminated by *Campylobacter* spp. at a frequency considerably higher than found in the other three types of meat studied because live chickens are known to be important

reservoirs of the organism (Hermans *et al.*, 2011). The isolation rate of 19.0% for *Listeria* spp. is again lower than those reported by others, 27.2% by Akya *et al.* (2010) and the 48% by Soultos *et al.* (2003). The 3.3% prevalence found for *Salmonella* spp. in our study is slightly lower than the 5.1% reported for chickens (Madden *et al.*, 2011) and considerably lower than the 80.0% reported in another study (Ananchaipattana *et al.*, 2012). Regardless, the isolation of these three pathogens on meat samples tested in the current study emphasizes the food safety concerns they pose to consumers (Borch and Arinder, 2002). The rate of isolation (25.0%) for *Proteus spp.* is also low when compared with the 53.9% (Tassew *et al.*, 2010) and the 55% (Lucia *et al.*, 1993) reported in other studies, yet their presence on the meat samples poses spoilage potential which has economic consequences.

It is imperative that to perform valid assays, there must be absence of PCR inhibition as demonstrated in our study. Conventional and TaqMan-PCR have been validated by others and have been used for simultaneous detection of these three organisms and several food borne pathogens (Cremonesi *et al.*, 2014; Suo *et al.*, 2010). The amplification and validation of PCR assays before being used for detection of food borne pathogens cannot be over-emphasized to eliminate false-positive or false-negative test results.

The detection of *C. jejuni* using this procedure might have amplified viable but non-cultivable (VBNC) bacteria that do not form colonies on culture media (James, 2005) while limiting any associated PCR inhibition. The author demonstrated that *C. jejuni* made VBNC in water caused death in suckling mice, still posing a potential public health hazard (James, 2005). In the current study this phenomenon was not observed with both *S.* Typhimurium and *L. monocytogenes*.

It is of diagnostic importance that the detection rates were the same by both the PCR-C and BIOLOG technique for the three pathogens tested. This is important because both assays were performed in
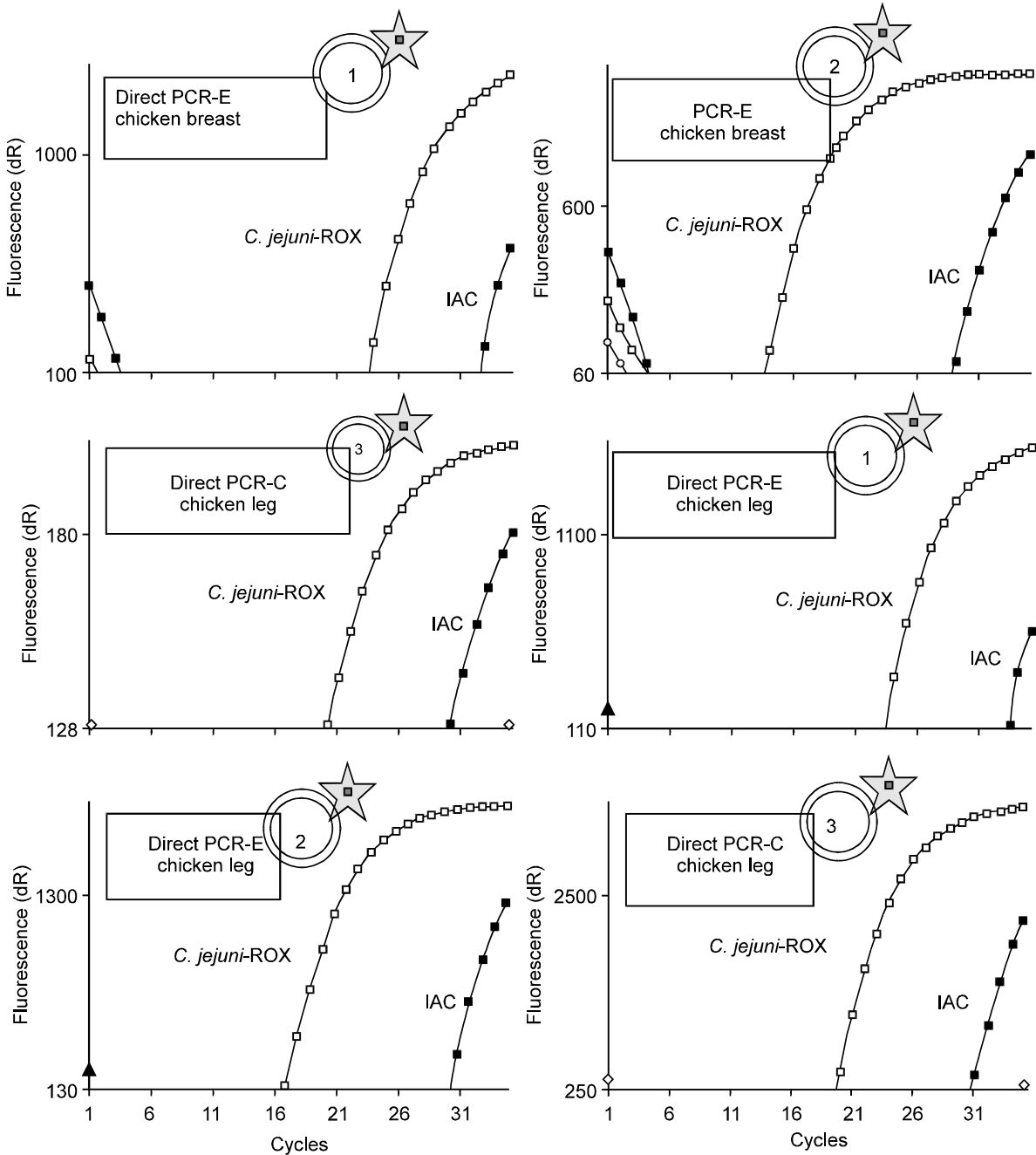
Fig. 4: Graphs of TaqMan PCR assays of *C. jejuni* using three types of PCR performed in this study. Direct PCR-E represent direct amplification from Campylobacter enrichment broth without DNA extraction, PCR-E represent amplification from concentration by centrifugation followed by DNA extraction and PCR-C represent amplification from DNA extracted from colony re-suspended in 1 ml tryptic soy broth. Both chicken breast and chicken leg samples provided early detection with PCR-E method and associated lower Ct values for IAC and *C. jejuni* represent absence or limited PCR inhibition with this method

parallel on the same meat samples to detect natural contamination by these pathogens. It is however pertinent to mention that reports exist on the higher rate of detection of food pathogens by PCR assays than the conventional culture methods (Kawasaki *et al.*, 2005, 2009, 2010). The sensitivity of conventional methods used in these studies cannot be ignored when being compared with PCR assays. Other studies have used
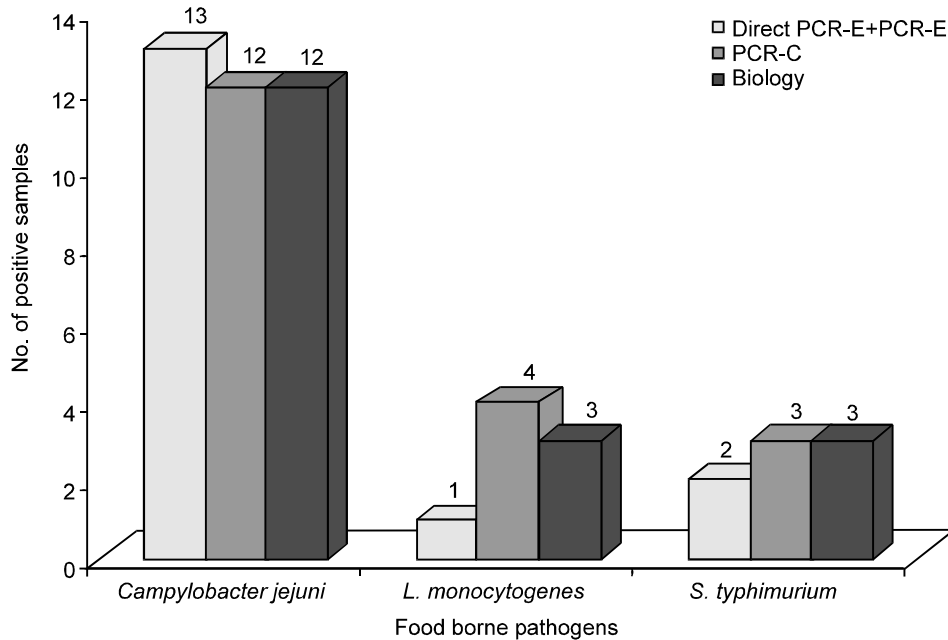
Biology/PCR-C*							
		Campylobacter jejuni		*Listeria monocytogenes*		*Salmonella typhimurium*	
		+	-	+	-	+	-
Direct PCR-E and PCR-E	+	8	5	1	0	2	0
	-	4	0	3	0	1	0
Agreement		8/17 (47%)		1/4 (25%)		2/3 (67%)	

*Agreement of biology idnetification and PCR-C was found to be 100%

Fig. 5: Detection of *C. jejuni*, *L. monocytogenes* and *S.* Typhimurium from meat samples by conventional PCR

PCR to detect pathogens on meat samples routinely spiked with selected pathogens to qualitatively and quantitatively detect their presence, as well as to confirm the sensitivity, specificity and accuracy of the assay systems (Cremonesi *et al.*, 2014; Sheare *et al.*, 2001; Suo *et al.*, 2010).

With the PCR system developed to detect the three pathogens in the current study, we achieved detection within 1-2 days compared with the conventional culture techniques, which took 6-7 days. Similar reports exist on the speed and accuracy of PCR compared to culture methods to detect food pathogens, at least 2 day earlier than the conventional culture methods (Kawasaki *et al.*, 2005; Kim *et al.*, 2007; Sheare *et al.*, 2001). The importance of rapid and accurate screening of meat destined for human consumption, albeit raw in this case, cannot be over-emphasized.

With *C. jejuni* where far more samples were positive compared with the other two pathogens, it was a surprise to have detected that as high as 29.4% of the samples were PCR-E positive but PCR-C negative. This finding may be explained, in part, by VBNC *C. jejuni* cells that may not have resumed growth even when nutrients

were provided, as earlier demonstrated by Nystrom (Nystrom, 2001). The detection of 23.5% PCR-E negative but PCR-C positive samples of *C. jejuni* could not be easily explained because the PCR-E protocol used in this study utilized 1 ml aliquot of the 45 ml homogenized sample which should be enough for detection by PCR. Finally, the finding of 100% agreement amongst BIOLOG, PCR-C methods and TaqMan assays for the three pathogens was expected since all the three organisms were specifically identified using recommended biochemical tests and amplification of their respective specific genome region provided a perfect correlation of the results of both assays.

The primary objective of any new method, particularly the PCR assays, is to provide accurate and rapid results to facilitate prompt intervention before contaminated foods reach consumers. That was the rationale of the current study to determine the presence of the pathogens by the three methods (PCR-E, PCR-C and Biolog). Using the assay we developed, the numbers of positive samples contaminated by the three bacteria were similar (Fig. 5), an indication that PCR-E and PCR-C may be comparable.
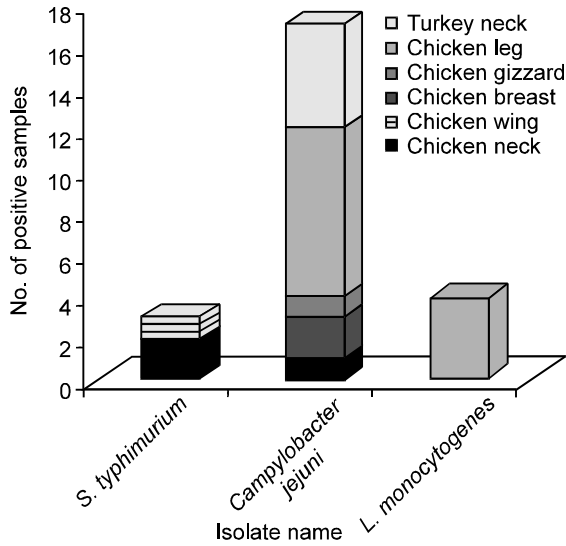
Fig. 6: Comparison of detection of *C. jejuni*, *L. monocytogenes* and *S.* Typhimurium by PCR performed on enrichment growth (PCR-E), PCR done on culture (PCR-C) and using conventional culture method (Biolog)

Data obtained in the current study also demonstrate that the sensitivity of culture method (BIOLOG) was similar to that of PCR (PCR-E or PCR-C).

A limitation of the study is that the PCR assay developed was specific for only *S.* Typhimurium with the implication that other serotypes of *Salmonella* that may be present in meat will remain undetected. Efforts are underway to develop a similar assay that will simultaneously detect a number of prevalent serotypes of *Salmonella* implicated in food borne outbreaks.

It is concluded that the PCR developed as a quick diagnostic tool in the current study reveal the presence of three important pathogens in packed ready-to-cook retail meat samples. The presence of the three pathogens in these meat samples, coupled with the rather high prevalence of *C. jejuni*, is considered a human health hazard, should the contaminated meat not be subjected to adequate heat treatment prior to consumption.

ACKNOWLEDGEMENTS

Disclosure statement: No competing financial interests exist.

REFERENCES

Adedayo, O. and B. Kirkpatric, 2008. *Campylobacter jejuni* Infections: Update on Presentation, Diagnosis and Management. Hospt. Phys., 44: 9-15.

Akya, A., A. Najafi, J. Moradi, Z. Mohebi and S. Adabaragher, 2010. Prevalence of food contamination with Listeria spp. in Kermanshah, Islamic Republic of Iran. Eastern Mediterr. Hlth. J., 19: 474-477.

Almeida, G., A. Morvan, R. Magalhaes, I. Santos, T. Hogg, A. Leclercq and P. Teixeira, 2010. Distribution and characterization of Listeria monocytogenes clinical isolates in Portugal, 1994-2007. Eur. J. Clin. Microbiol. Infect. Dis., 29: 1219-1227.

Ananchaipattana, C., Y. Hosotani, S. Kawasaki, S. Pongsawat, B.M. Latiful, S. Isobe and Y. Inatsu, 2012. Prevalence of foodborne pathogens in retailed foods in Thailand. Foodborne Pathog. Dis., 9: 835-840.

Bohaychuk, V.M., G.E. Gensler, R.K. King, K.I. Manninen, O. Sotrnsen, J.T. Wu, M.E. Stiles and L.M. McMullen, 2006. Occurrence of pathogens in raw and ready-to-eat meat and poultry products collected from the retail marketplace in Edmonton, Alberta, Canada. J. Food Prot., 69: 2176-2182.

Borch, E. and P. Arinder, 2002. Bacteriological safety issues in red meat and ready-to-eat meat products, as well as control measures. Meat Sci., 62: 381-390.

CDC, 2010. Estimates of Foodborne Illness in the United States. Vol. 2012, 2010.

CDC, 2011. National *Salmonella* Surveillance Overview. U.S Department of Health and Human Services, Atlanta. Georgia, 2011.

CDC, 2014. Outbreak of salmonellosis associated with consumption of pulled pork at a church festival-Hamilton County, Ohio, 2010. MMWR, 62: 1045-1047.

Cook, A., J. Odumeru, S. Lee and F. Pollari, 2012. Campylobacter, *Salmonella*, Listeria monocytogenes, verotoxigenic *Escherichia coli* and *Escherichia coli* prevalence, enumeration and subtypes on retail chicken breasts with and without skin. J. Food Prot., 75: 34-40.

Cremonesi, P., L.F. Pisani, C. Lecchi, F. Ceciliani, P. Martino, A.S. Bonastre, A. Karus, C. Balzaretti and B. Castiglioni, 2014. Development of 23 individual TaqMan (R) real-time PCR assays for identifying common foodborne pathogens using a single set of amplification conditions. Food Microbiol., 43: 35-40.

Fakruddin, M., K.S. Mannan and S. Andrews, 2013a. Viable but nonculturable bacteria: food safety and public health perspective. ISRN Microbiol., 703813.

Fakruddin, M.D., M. Sultana, M.M. Ahmed, A. Chowdhury and N. Choudhury, 2013b. Multiplex PCR (polymerase chain reaction) assay for detection of *E. coli* O157:H7, *Salmonella* sp., Vibrio cholerae and Vibrio parahaemolyticus in spiked shrimps (Penaeus monodon). Pak. J. Biol. Sci., 16: 267-274.

FSIS, 2009. Isolation and identification of Listeria monocytogenes from red meat, poultry, egg and environmental samples. Available at http://www.fsis. usda.gov/PDF/MLG-8.pdf.

Gallagher, D., E.D. Ebel, O. Gallagher, D. Labarre, M.S. Williams, N.J. Golden, R. Pouillot, K.L. Dearfield and J. Kause, 2013. Characterizing uncertainty when evaluating risk management metrics: risk assessment modeling of Listeria monocytogenes contamination in ready-to-eat deli meats. Int. J. Food Micobiol., 162: 266-275.

Hermans, D., Van K. Deun, A. Martel, Van F. Immerseel, W. Messens, M. Heyndrickx, F. Haesebrouck and F. Pasmans, 2011. Colonization factors of *Campylobacter jejuni* in the chicken gut. Vet. Res., 42: 82.

Hunt, J.M., C. Abeyta and T. Tran, 2013. Campylobacter. In: Bacteriological Analystical Manual, (FDA, ed.), MD., USA.

James, D.O., 2005. The Viable but Nonculturable State in Bacteria. J. Microbiol., 43: 93-100.

Karch, H., E. Denamur, U. Dobrindt, B.B. Finlay, R. Hengge, L. Johannes, E.Z. Ron, T. Tonjum, P.J. Sansonetti and M. Vicente, 2011. The enemy within us: lessons from the 2011 European *Escherichia coli* O104:H4 outbreak. EMBO Mol. Med., 4: 841-848.

Kawasaki, S., P.M. Fratamico, N. Horikoshi, Y. Okada, K. Takeshita, T. Sameshima and S. Kawamoto, 2009. Evaluation of a multiplex PCR system for simultaneous detection of *Salmonella* spp., Listeria monocytogenes and *Escherichia coli* O157:H7 in foods and in food subjected to freezing. Foodborne Path. Dis., 6: 81-89.

Kawasaki, S., P.M. Fratamico, N. Horikoshi, Y. Okada, K. Takeshita, T. Sameshima and S. Kawamoto, 2010. Multiplex real-time polymerase chain reaction assay for simultaneous detection and quantification of *Salmonella* species, Listeria monocytogenes and *Escherichia coli* O157:H7 in ground pork samples. Foodborne Pathog. Dis., 7: 549-554.

Kawasaki, S., N. Horikoshi, Y. Okada, K. Takeshita, T. Sameshima and S. Kawamoto, 2005. Multiplex PCR for simultaneous detection of *Salmonella* spp., Listeria monocytogenes and *Escherichia coli* O157:H7 in meat samples. J. Food Prot., 68: 551-556.

Kim, H.J., J.C. Cho, H.J. IM and J.C. Cho, 2008. Rapid and sensitive detection of Listeria monocytogenes using a PCR-Enzyme-linked immunosorbent assay. J. Microbiol. Biotech., 18: 1858-1861.

Kim, J.S., G.G. Lee, J.S. Park, Y.H. Jung, H.S. Kwak, S.B. Kim, Y.S. Nam and S.T. Kwon, 2007. A novel multiplex PCR assay for rapid and simultaneous detection of five pathogenic bacteria: *Escherichia coli* O157:H7, *Salmonella*, Staphylococcus aureus, Listeria monocytogenes and Vibrio parahaemolyticus. J. Food Prot., 70: 1656-1662.

Lamden, K.H., A.J. Fox, C.F. Amar and C.L. Little, 2013. A case of foodborne listeriosis linked to a contaminated food-production process. J. Med. Microbiol., 62: 1614-1616.

Lee, N., K.Y. Kwon, S.K. Oh, H.J. Chang, H.S. Chun and S.W. Choi, 2014. A Multiplex PCR Assay for Simultaneous Detection of *Escherichia coli* O157:H7, Bacillus cereus, Vibrio parahaemolyticus, *Salmonella* spp., Listeria monocytogenes and Staphylococcus aureus in Korean ready-to-eat food. Foodborne Pathog. Dis., 11: 574-580.

Lucia, L.M., C. Vanderzant and G.R. Acuff, 1993. Potential role of Proteus-Providencia-Morganella species in quality deterioration of beef. Meat Sci., 35: 321-330.

Madden, R.H., L. Moran, P. Scates, J. McBride and C. Kelly, 2011. Prevalence of Campylobacter and *Salmonella* in raw chicken on retail sale in the republic of Ireland. J. Food Protec., 74: 1912-1916.

Mercado, M., J. Avila, M. Rey, M. Montoya, A.K. Carrascal and D.X. Correa, 2012. Outbreaks of *Salmonella* spp., Staphylococcus aureus and Listeria monocytogenes associated with poultry consumption. Systematic review]. Biomedica: Rev. del Instituto Nacional de Salud, 32: 375-385.

Much, P., J. Pichler and F. Allerberger, 2007. Food borne infectious outbreaks, Austria 2005. Wien Klin. Wochenschr, 119: 150-157.

Nystrom, T., 2001. Not quite dead enough: on bacterial life, culturability, senescence and death. Arch. Microbiol., 176: 159-164.

Phillips, D., D. Jordan, S. Morris, I. Jenson and J. Sumner, 2008. A national survey of the microbiological quality of retail raw meats in Australia. J. Food Protec., 71: 232-1236.

Raguenaud, M.E., Le S. Hello, S. Salah, F.X. Weill, A. Brisabois, G. Delmas and P. Germonneau, 2012. Epidemiological and microbiological investigation of a large outbreak of monophasic *Salmonella* Typhimurium 4,5,12:i:- in schools associated with imported beef in Poitiers, France, October 2010. Euro surveillance: bulletin Europeen sur les maladies transmissibles. Euro. Comm. Dis. Bull., 17: 20289.

Rokosz, N., W. Rastawicki and T. Wolkowicz, 2014. [Microbiological diagnosis of infections caused by Campylobacter jejuni and Campylobacter coli in humans. Postepy higieny i medycyny doswiadczalnej, 68: 48-56.

Sammarco, M.L., G. Ripabelle, G. Fanelli, G.M. Grasso and M. Tamburro, 2010. Prevalence and biomolecular characterization of Campylobacter spp. isolated from retail meat. J. Food Prot., 73: 720-728.

Scallan, E., R.M. Hoekstra, F.J. Angulo, R.V. Tauxe, M.A. Widdowson, S.L. Roy, J.L. Jones and P.M. Griffin, 2011. Foodborne illness acquired in the United States-major pathogens. Emerg. Infect. Dis., 17: 7-15.

Sheare, A.E., C.M. Strapp and R.D. Joerger, 2001. Evaluation of a polymerase chain reaction-based system for detection of *Salmonella* enteritidis, *Escherichia coli* O157:H7, Listeria pp. and Listeria monocytogenes on fresh fruits and vegetables. J. Food Prot., 64: 788-795.

Shu, B., C. Zhang and D. Xing, 2014. Segmented continuous-flow multiplex polymerase chain reaction microfluidics for high-throughput and rapid foodborne pathogen detection. Analyt. Chim. Acta, 826: 51-60.

Sison, F.B., W. Chaisowwong, T. Alter, S. Tiwananthagorn, D. Pichpol, K.N. Lampang, M.P. Baumann and G. Golz, 2014. Loads and antimicrobial resistance of Campylobacter spp. on fresh chicken meat in Nueva Ecija, Philippines. Poultr. Sci., 93: 1270-1273.

Soultos, N., P. Koidis and R.H. Madden, 2003. Presence of Listeria and *Salmonella* spp. in retail chicken in Northern Ireland. Lett. Appl. Microbiol., 37: 421-423.

Suo, B., Y. He, S.I. Tu and X. Shi, 2010. A multiplex real-time polymerase chain reaction for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157 and Listeria monocytogenes in meat products. Foodborne Pathog. Dis., 7: 619-628.

Suo, B., Y. Wang, B. UO and Y. Wang, 2013. Evaluation of a multiplex selective enrichment broth SEL for simultaneous detection of injured *Salmonella*, *Escherichia coli* O157:H7 and Listeria monocytogenes. Brazilian J. Microbiol., 44: 737-742.

Tassew, H., A. Abdissa, G. Beyene and S. Gebre-Selassie, 2010. Microbial flora and food borne pathogens on minced meat and their susceptibility to antimicrobial agents. Ethiopian J. Hlth. Sci., 20: 137-143.

Wilson, D.J., E. Gabriel, A.J.H. Leatherbarrow, J. Cheesbrough, S. Gee, E. Bolton, A. Fox, P. Fearnhead, C.A. Hart and P.J. Diggle, 2008. Tracing the Source of Campylobacteriosis. PLoS Genet., 4: e1000203.

Woubit, A., T. Yehualaeshet, T. Habtemariam and T. Samuel, 2012. Novel genome tools for specific and real-time detection of biothreat and frequently encountered foodborne pathogens. J. Food Prot., 75: 660-670.