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Use of *invA* Gene Specific PCR Analysis for the Detection of Virulent *Salmonella* Species in Beef Products in the North West Province, South Africa

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Abstract A total of 32 beef samples were collected from shops around the North West Province, South Africa and analyzed for the presence of *Salmonella* species. A total of 96 presumptive isolates were screened for characteristics of *Salmonella*. All (100%) of the isolates were Gram negative rods and catalase positive. However, only a small proportion (25%) utilized citrate as a sole carbon source while 78% of the isolates fermented the sugars glucose, lactose and sucrose. Gas was produced by 34.4% while only 3.1% of these isolates produced hydrogen sulphide gas. Despite the fact that a small proportion (12.5%) of these isolates were identified as *Salmonella* species by the API 20E test, a much larger proportion agglutinated with *Salmonella* Poly A-S antiserum (53.1%) when compared to the *Salmonella* Poly O antiserum. To avoid bias, all the 96 isolates were screened for the *Salmonella* specific *invA* gene through PCR analysis and only 10 (10.4%) isolates were positively identified. Moreover, none of the isolates possessed the *fliC* flagella gene while a small proportion 11(11.5%) were positive for the *fljB* gene fragments. The presence of these pathogenic *Salmonella* species in raw meat indicates the health risk that these food products can cause to consumers, especially if consumed undercooked.

Keywords: *Salmonella species, beef, invA, fliC, fljB, virulence gene*

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1. Introduction

Salmonella species exist as part of the normal flora of cattle or as a transient members of the gastrointestinal tract of humans and warm blooded animals [1]. They are therefore released to the environment if proper sanitary practices are not implemented. The hides of animals including cattle usually possess high numbers of microbial pathogens and these contaminants can be transmitted to the carcass of animals during slaughter [2-8]. Usually contamination of beef carcasses during slaughter is almost unavoidable and the removal of intestinal contents may play a critical role in the transmission of *Salmonella* species to the carcass [9]. Despite the fact that *Salmonella* species have been isolated from a number of animal species [10,11], cattle are known to be the main reservoir and therefore these organisms are often isolated from carcass of healthy cattle in abattoirs [1,8,12,13]. Raw meat has enriched nutrient composition, water activity of 0.98-0.99, and pH ranging from 5.5 to 6.5 and this supports growth of most contaminating microorganisms [14]. Unfortunately, the presence of microbial contaminants in meat products cannot be detected visually [15] this increases both the risks associated with foodborne pathogens and the incidence of human diseases.

Individuals living in developing countries including South Africa are affected with episodes of approximately 1.5 billion foodborne diseases yearly [16] and *Salmonella* species are currently documented to be the most frequently isolated pathogen in a large proportion of the outbreaks of infections worldwide [17-23]. Infections caused by *Salmonella* species in humans usually result from the consumption of contaminated food products [17,24,22,25]. Undercooked beef, and poultry have been reported to cause sporadic cases and outbreaks of infections in many parts of the world [26,27,28,29,30]. Lack of proper sanitary practices by food handlers is also a significant cause of cross contamination [31,32]. Even though proper cooking may destroy and inactivate these pathogens, individuals who serve at retail points or ready to eat food outlets must ensure that cross-contamination is greatly minimized. Moreover, the storage of meat at high ambient temperatures and humidity including contact with contaminated surfaces increase the opportunities for massive microbial contamination and greatly enhances deterioration [33,34].

Salmonella species are responsible for a large degree of mortality and morbidity worldwide [19,20,21]. Infections caused by these organisms range from non-bloody

diarrhea that is usually self-limiting to salmonellosis, mild fever, nausea, abdominal pains, enteric (typhoid) fever, bacteremia, septicemia and gastroenteritis in humans [35,36,37]. Among these, salmonellosis accounts for the major health, financial and socioeconomic burden caused by *Salmonella* species in infected patients worldwide [38,39,40,41]. Despite this, it is usually difficult to evaluate the direct contribution *Salmonella* species in foodborne infections in developing countries such as South Africa. Reasons include the lack of epidemiological pathogen surveillance systems, under-reporting of cases, limited documented studies and the fact that there are a wide variety of pathogens that are also known to cause foodborne infections in humans [42,43,44,45]. In spite of these, there is limited data on the occurrence of *Salmonella* species in cattle, their carcasses in abattoirs and other food product in the North West Province, South Africa [46]. Given that contamination of raw meat results from a variety of pre-harvest, harvest and post-harvest processes [47,48], bacterial pathogens including *Salmonella* pose severe meat safety concerns to consumers [49]. Moreover, since the microbiological quality of meat depends on the control measures implemented during slaughter [2,50,51] it is therefore important to generate data on the level of carcass contamination during slaughter. This may provide options for mitigation strategies and also address the risks associated with *Salmonella* contamination in the food industry. Therefore the present study was designed to isolate *Salmonella* species from retail beef that is sold in supermarkets in North West Province in South Africa using conventional and PCR assays.

2. Materials and Methods

2.1. Area of Study

The research was conducted in the North West University- Mafikeng Campus, North West Province, South Africa. A total of 32 meat samples were obtained from shops and some supermarkets in the North West Province, South Africa. Table 1 indicates the number of meat samples collected from different sample sites.

Table 1. Areas from which beef (meat) samples were collected

District	Sampling Area	Number of samples
Dr. Kenneth Kaunda	Carltonville	7
	Potchefstroom	3
Dr Ruth Mompati	Ganyesa	1
	Vryburg	2
Dr Modiri Molema	Coligny	1
	Dingateng	1
	Lichtenburg	2
	Logagane	1
	Mabule	2
	Mafikeng	12

2.2. Sample Collection

A total of 32 beef samples were collected randomly from retail shops/ supermarkets in different towns and villages around the North West province South Africa. The samples were properly labeled, kept in their original packaging and transported to the laboratory on ice for analysis.

2.3. Isolation of *Salmonella* Species

Laboratory analysis of meat samples was achieved by washing 1g of meat in 5 mL Rappaport Vassiliadis (RV) broth. The sample was incubated at 37°C for 24 hours to enrich for *Salmonella* species. A loopful of the homogenate was streak on *Salmonella–Shigella* agar (SS-agar) and plates were incubated at 37°C for 24 hours. Characteristic pink or yellow colonies with or without black centers on SS-agar were picked, purified by sub-culturing on SSA and used for bacterial identification tests.

2.4. Preliminary Bacterial Identification

Presumptive isolates were identified using the following criteria:

2.4.1. Gram Staining

All isolates were Gram stained using standard technique [52]. After staining slides were observed under oil immersion for the presence of Gram negative rod shaped cells.

2.4.2. Catalase Test

The catalase test facilitates the detection of the catalase enzyme in bacteria containing the cytochrome system. The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. The test was performed using 2% (v/v) hydrogen peroxide. A pure colony of the isolate was placed on a clean microscope slide and a drop of 2% (v/v) was added to the culture. The formation of bubbles indicated a positive result and vice versa.

2.4.3. Triple Sugar Iron (TSI) Agar Test

All presumptive isolates were subjected to the Triple sugar iron (TSI) agar test (Biolab, Merck - South Africa) This test was utilized to assay *Salmonella* isolates against three sugars (glucose, lactose and sucrose) present at concentrations 0.1%, 1.0% and 1.0% respectively. The test was performed according to standard protocols. A pure colony was stab-inoculated into to the butt of the TSI medium and later streaked on the slant. The bottle was loosely closed and incubated aerobically at 37°C for 24 hours. A change in colour from pink to yellow indicated that the sugars had been fermented and results were interpreted based on standard protocols.

2.4.4. Simmons Citrate agar Test

This test is based on the ability of Enterobacteriaceae to utilize citrate as the sole source of carbon. A pure colony was stab-inoculated into to the butt of the Simmons Citrate medium and later streaked on the slant. The bottle was loosely closed and incubated aerobically at 37°C for 24 hours. A colour change from green to blue indicated positive results which were interpreted using standard protocols.

2.5. Confirmatory Biochemical Identification Tests for Isolates

2.5.1. Analytical Profile Index (API 20E) test

The identities of all presumptive *Salmonella* species were confirmed using analytical profile index API 20E

test. The test was performed according to manufacturer's protocol (BioMerieux, Marcy l'Etoile, France). The results were read with reagents and the indices based on phenotypic profiles of isolates were used to determine the identity of isolates using the API web software.

2.5.2. Serological Test

Serological tests were performed using the rapid slide agglutination assay with *Salmonella* –specific Poly A-S and Poly O antisera according to manufacturer's protocol (Mast Diagnostics, (Neston, Wirral, UK). A drop of saline was placed on a glass slide and a pure colony was obtained using a wire loop and placed on the saline drop and mixed well. One drop of *Salmonella* specific antisera was added and mixed. Agglutination that was visible after a minute was recorded as positive.

2.6. Molecular Characterization of Isolates

2.6.1. Extraction of Genomic DNA

Genomic DNA was extracted from all *Salmonella* isolates using a DNA extraction kit (Zymo genomic DNA-Tissue MiniPrep) according to manufacturer's protocol (Zymo Research Corporation USA). The DNA extracted was stored at 4°C until PCR analysis.

2.6.2. *Salmonella* Specific PCR Assay

The identities of presumptive *Salmonella* isolates were confirmed through amplification of the *invA*, *fljB* and *fliC* gene fragments. The primer pair invAF (5'-GTGAAATTATCGCCACGTTCGGGCAA-3') and invAR (5'-TCATCGCACCGTCAAAGGAACC -3') were used to amplify the *invA* gene fragment that is specific to *Salmonella* species [53]. Primers fliCF (5'-CACTGGTCTTAATGATGCAGCTC-3') and fliCR (5'-CCTGTCACTTTCGTGGTTAT-3') were also used to amplify the *fliC* gene fragment while fljBF (5'-GGCAACCCGACAGTAACTGGCGATC-3') and fljBR (5'-ATCAACGGTAACTTCATATTTG-3') were used to make copies of the *fljB* gene. PCR reactions were performed standard 25µL volumes containing 2µL of DNA template, 12.5µL PCR master mix, 1.5µL PCR reaction buffer, 0.5µL of both oligonucleotide primers and 8.5µL nuclease free water. The primers were synthesized by Inqaba Biotec Pty Ltd, Pretoria, South Africa while all other reagents were obtained from Fermenters, South Africa. PCR amplification was performed with a DNA thermal cycler (Model – Bio- RAD C1000 Touch ™ Thermal Cycler) using the following conditions: 94°C for 600 seconds, followed by 46 cycles of 94°C for 30 seconds, 41°C for 60 seconds, 72°C for 120 seconds and a final extension at 72°C for 5 300 seconds.

2.7. Electrophoresis of PCR Products

PCR products were resolved by agarose gel (2% w/v) electrophoresis on a horizontal Pharmacia biotech equipment system (model Hoefer HE 99X; Amersham Pharmacia biotech, Sweden). Gels were run for 5 hours at 60 V using 1X TAE buffer (40mM Tris, 1mM EDTA and 20nM glacial acetic acid, pH 8.0). Each gel contained a 100bp DNA molecular weight maker (Fermenters, USA). Ethidium Bromide (0.1µg/ml) was used to stain the gels for 15 minutes. Amplicons were visualized under U.V light at 420 nm [54]. A Gene Genius Bio Imaging System (Syngene, Synoptics; UK) was used to capture the image using GeneSnap (version 6.00.22) software. Gene Tools (version 3.07.01) software (Syngene, Synoptics; UK) was used to analyze the images in order to determine the relative sizes of the amplicons.

3. Results and Interpretation

3.1. Detection of *Salmonella* Isolates in Retail Beef Samples Using Preliminary and Confirmatory Biochemical Tests

A total of 32 beef samples were collected and analyzed for the presence of *Salmonella* species using preliminary (Gram staining, catalase test, Simmons citrate agar test, Triple sugar iron agar test) and confirmatory tests (serological test and specific PCR). The proportion of isolates that satisfied these tests are shown in Table 2. A total of 96 isolates were screened for characteristics of *Salmonella* and all (100%) of the isolates were Gram negative rods and catalase positive. Of these isolates 25% were able to utilize citrate as a sole carbon source. However, a large proportion (78%) of the isolates were able to ferment sugars in the TSI medium, 34.4% were able to produce gas while only 3.1% produced hydrogen sulphide gas. A small proportion of these isolates 12.5% were identified as *Salmonella* species using API 20E. However, a large proportion (53.1%) of the isolates tested positive for the *Salmonella* Poly A-S antiserum while 34.4% were positive for Poly O antiserum. Despite the fact that *Salmonella* species was detected by serotyping among isolates from Carltonville, Lichtenburg, Dingateng and Ganyesa none of these isolates were identified by API 20E with samples from Ganyesa (Table 2).

Table 2. Results for Preliminary and Confirmatory Biochemical tests

SS	GS(+ rod)	CT (+)	CU (+)	API 20E	ST		TSI		
					Sal Poly A-S	Sal Poly O	C	G	H
CA	100	100	42.9	0	57.1	28.6	71.4	42.9	0
CO	100	100	0	100	100	100	100	0	0
VEN	100	100	0	0	0	100	0	0	0
LIC	100	100	0	0	50	0	0	0	0
POT	100	100	0	33.3	33.3	33.3	0	0	0
MAF	100	100	25	16.7	50	33.3	100	33.3	0
DIN	100	100	0	0	100	100	100	100	100
LOG	100	100	100	0	100	0	100	0	0
MAB	100	100	50	0	50	50	100	50	0
VRY	100	100	0	0	0	0	100	50	0
GAN	100	100	0	0	0	0	100	100	0

CA=Carltonville, CO=Coligny, VEN=Ventersdorp, LIC=Lichtenburg POT=Potchefstroom, MAF=Mafikeng, DIN=Dingateng, LOG=Logagane, MAB=Mabule, VRY=Vryburg, GAN=Ganyesa.

3.2. PCR for the Detection of Invasive *Salmonella* Species Specific Gene

To avoid bias all the 96 isolates were also subjected to specific PCR analysis for characters of *Salmonella* species using a specific primer sequence. This was achieved through amplification of the *invA* gene fragments specific for *Salmonella* isolates. Figure 1 shows a composite picture of a 2% (w/v) agarose gel of the *invA* gene fragments amplified from the isolates. A summary of the isolates that were positive for the *invA Salmonella* specific PCR are shown in Table 3. As shown in Table 3, only 10 (10.4%) isolates were identified as *Salmonella* species and a majority of the positive isolates were from Carletonville (38%) followed by Dingateng, Logagane, Vryburg and Ganyesa.

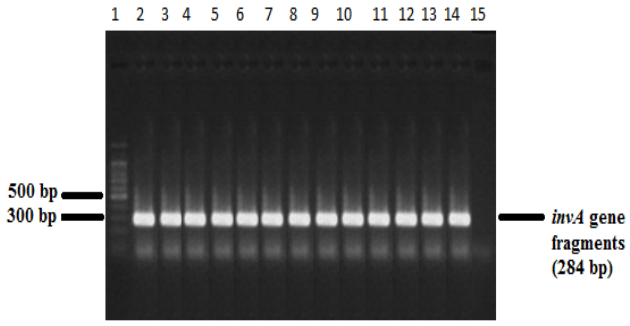

Figure 1. PCR for the detection of the *invA* gene fragments amplified from *Salmonella* isolates. Lane 1= 100bp marker; Lanes 2-14= *invA* gene amplified from *Salmonella* isolates obtained from beef samples; Lane 15=Negative control (No template DNA) sample

3.3. PCR for the Identification of *Salmonella* Isolates Using *fljB* and *fliC* Primers

The isolates that were positive for the *invA* gene PCR analysis were further subjected to specific PCR analysis to detect the presence of two flagella gene fragments specific for *Salmonella* species. This involved amplification of the *fljB* and *fliC* gene fragments that form part of virulence factors of *Salmonella* species. Despite the fact that none of the isolates possessed the *fliC* genes, a small proportion 11(11.5%) were positive for the *fljB* gene fragments. Detailed results are shown in Table 3.

Table 3. Proportion of *Salmonella* isolates that were positive for the *invA*, *fljB* and *fliC* genes

Sample site	No of isolates tested	Percentage of isolates positive for the targeted genes		
		invA	*fljB*	*fliC*
Carltonville	21	38	28.6	0
Coligny	3	0	33.3	0
Ventersdorp	3	0	0	0
Lichtenburg	6	0	16.7	0
Potchefstroom	9	22.2	11.1	0
Mafikeng	36	0	0	0
Dingateng	3	33.3	33.3	0
Logagane	3	33.3	0	0
Mabule	6	16.7	0	0
Vryburg	6	33.3	28.6	0
Ganyesa	3	33.3	33.3	0

4. Discussions

Salmonellosis places significant health and economic burden on humans worldwide [41]. The impact is higher in developing countries that do not have access to appropriate public health facilities and individuals do not practice proper sanitation. *Salmonella* species have been implicated as a common cause of foodborne illnesses which is frequently associated with diarrhea, nausea, mild fever and abdominal pains [17-23,35]. Outbreaks of human infections have been the major trigger resulting in intensified efforts to improve surveillance of the pathogen in the farms; hygiene standards in slaughter facilities and even in retail shops [55]. Raw and under cooked beef and poultry have been recognized as significant sources of human salmonellosis [27,28,29,30]. In spite of this, there is very little documented report on the occurrence of *Salmonella* species in food products in the North West Province, South Africa [46]. It is therefore hypothesized that consumption of undercooked beef that is contaminated with faeces from infected animals may serve as potential vehicle for transmission of these pathogens to humans.

In this study all the isolates from different sampling sites were both Gram negative rod shaped bacteria and catalase positive. However, a small proportion (24%) of the all the isolates utilized citrate as a sole carbon source. In contrast only 10.4% of these isolates were positively identified as *Salmonella* species using API20E assay. These included isolates from Coligny (100%), Potchefstroom (33.35%) and Mafikeng (16.7%) respectively. In this study 50% of the isolates were positive for *Salmonella* Poly A-S antiserum, 34.4% were positive for *Salmonella* Poly O antiserum while 75% were positive for both *Salmonella* Poly A-S and Poly O antiserum respectively. Moreover, 12.5% of these isolates were positive for the *invA* gene. Similar studies have been conducted to determine the occurrence of *Salmonella* species in hide, faecal matter, bovine carcasses in abattoirs and retail beef [11,12,13,15,28,48,50,56,57] using either few or a combination of methods utilized in our study. In one study, no *Salmonella* species was detected in bovine carcasses using biochemical assays [15]. Moreover, in some other studies, the prevalence in beef carcass at different stages of processing ranged from 0.8% to 3.4% [12] and 0% to 2.94% in retail meat and meat products [48]. However, results obtained in studies conducted in Mexico indicated that the prevalence of *Salmonella* species in beef carcass decreased from 49% to 6% between the evisceration and dry chilling stages [12]. Similarly, a prevalence rate of 50.2% was reported in bovine carcass in the United States [58]. In addition prevalence rates of 63% and 43% were reported in beef carcasses and as high as 87% in retail beef in Dakar, Senegal [28]. Despite the fact that prevalence rates on the carcass may vary from one country to another and between reports in a given location, the microbiological quality of meat depends on the control measures implemented during slaughter [2,50,51]. It is suggested that the presence of *Salmonella* species in carcass may facilitate its transmission to the finished retail product. In addition the prevalence of *Salmonella* in retail meat in our study (12.5%) is even higher than previous reports [11,48]. Similar observations have been documented for

Salmonella species isolated from retail beef samples through amplification of the *invA* gene fragment [28,29,30,59]. In a previous study, *Salmonella* species were recovered from fresh beef 23.3% (21/90), ground beef 20% (18/90) and beef burger 12.2% (11/90) samples, respectively [30]. A comparative study involving fresh chicken and beef meat samples obtained at retail outlets in Tehran, Iran revealed that the prevalence of *Salmonella*, (33%) was higher than that of *Campylobacter* (29%) and *Yersinia* (16%) [29]. The detection of *Salmonella* species in retail meat in the North West Province, South Africa calls for attention and therefore indicates the need for reinforcing control measures.

Foodborne isolates may even present more challenging complications if they harbour virulence gene determinants. In the present study, none of the isolates were positive for the *fliC* genes. On the contrary, the *fljB* and the *invA* gene fragments were detected in 11.5% and 12.5% of the isolates. Similar observations have been reported [60] while in another study all the isolates possessed the *invA* gene [48]. It has been shown that the *invA* gene PCR is highly sensitive for the detection of *Salmonella* species and currently this genetic determinant is widely utilized in the detection of bacteria belonging to this genus [59].

Our findings demonstrated that the retail beef products tested were contaminated with virulent *Salmonella* species and such contamination may constitute a major public health concern since it can be transmitted through the food chain. A comparison of the genetic relationships of *Salmonella* species in slaughter houses and corresponding retail shops have been determined using pulsed-field gel electrophoresis (PFGE) and antimicrobial resistance patterns [28]. Results indicated that approximately 44% to 56% of *Salmonella* contaminants are equally shared by slaughterhouses and the distribution channels through improper handling and houseflies. In addition it has been shown that the serotype of *Salmonella* that was present in faeces and rumen of cattle was detected in beef carcasses and retail meat thereby posing direct threats to the health of consumers [56,61]. This highlights the need for appropriate hygiene practices to be implemented throughout the food production processes. The present baseline data on the prevalence *Salmonella* species on raw beef products of animal origin is needed to determine the extent of the problem and also to serve as a point of reference for monitoring food safety changes that occur in the area.

5. Conclusion

In the present study, Salmonella species were successfully isolated from the raw meat samples and some of the isolates possessed the *fljB* virulence gene fragments. These isolates may therefore pose severe health implications to humans if the meat products are not properly cooked before they are eaten.

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