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Isolation of Enterohaemorrhagic *Escherichia coli* O104 Strains from Raw Meat Products in the North West Province, South Africa

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Abstract Enterohaemorrhagic *E. coli* (EHEC) are Shiga toxin-producing *E. coli* strains that possess unique pathogenic properties and are characterized by certain seropathotypes that are frequently associated with outbreaks or sporadic cases of human infections. Although EHEC strains belonging to the serotype O104:H4 has rarely been associated with human diseases in the past, outbreaks of infections caused by this organism resulted to HUS and bloody diarrhoea in humans in countries that have advanced public health facilities. The aim of this study was to isolate *E. coli* O104 strains from meat samples obtained from supermarkets and retail shops in the North West Province, South Africa. A further objective was to determine the identities of the isolates using Gram staining, preliminary biochemical tests (oxidase test, Triple Sugar Iron (TSI) test, citrate utilization and Sorbitol fermentation test) and confirmatory (API 20E and PCR analysis) assays. A total of nineteen meat samples were collected from four butcheries and shops in the study area and Sorbitol MacConkey agar was used for selective isolation of bacteria. A total of 304 presumptive colonies were subjected to identification tests for *E. coli*. Large proportions (65.5% to 93%) of these isolates were oxidase negative; fermented the sugars in the TSI medium; utilized citrate and fermented sorbitol. On the contrary only a small proportion (27.3%) of the isolates produced hydrogen sulphide gas. Based on patterns obtained for the biochemical profiles of the isolates 102 (33.6%) were positively identified as *E. coli*. All the 304 *E. coli* isolates were subjected to specific PCR designed to amplify thewzx_{O104} gene fragment that facilitates identification of *E. coli* O104 strains and a total of 52 isolates were positively identified. Despite the fact that not all samples were positive for the pathogen, the presence of *E. coli* O104 strains in some of the meat samples was of great concern. These bacteria cells have the potential to cause severe health effects on consumers if present in undercooked food products.

Keywords: *Enterohaemorrhagic E. coli (EHEC), E. coli O104 strains, food contamination, beef, diarrhoea, wzxO104gene*

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1. Introduction

E. coli is a prominent member of the gastrointestinal tract of their hosts and are released into the environment by human and animal dejections when appropriate hygiene and sanitary practices are not implemented [1,2]. It is estimated that up 4% of all cultivable bacteria in the colon are *E. coli* and the fact that these bacteria species are most often present in the microbiota of the environment they are therefore used as indicators of faecal pollution [1,2,3]. Despite the fact that a number of non-pathogenic *E. coli* strains are known to maintain the physiological state of the gastrointestinal tract, enhance digestion and defend the host against other enteric pathogens, some strains possess and express a variety of virulence determinants [4,5,6]. Consequently, a number of *E. coli* strains are currently known to cause diseases ranging from diarrhoea, haemolytic colitis (HC), haemorrhagic uraemic syndrome (HUS) and septicaemia in humans [4,5,7,8,9]. Enterohaemorrhagic *E. coli* (EHEC) are Shiga toxin-producing *E. coli* strains that possess unique pathogenic properties [4], and are characterized by certain seropathotypes that are frequently associated with outbreaks or sporadic cases of human infections [10,11,12,13,14]. The host range of EHEC strains are domestic animals and colonized animals usually shed the bacteria through their faeces [15]. This therefore increases the chances of contamination [16] and the consumption of contaminated food of animal origin is known to be a source of human EHEC infections [17,18]. EHEC have thus been isolated from water, sewage, soil, manure, animal faeces, sprouts and fresh produce [12,15,19,20,21,22]. Despite the fact that contaminated

vegetables and sprouted seeds are considered the main vehicles for transmitting EHEC strains, meat and meat products have also been linked to some outbreaks caused by diarrheagenic *E. coli* in many parts of the world [23,24]. In South Africa and the North West Province in particular, *E. coli* O157:H7 strains have been extremely investigated mainly due to its severe concern resulting from the serious clinical outcomes of infections caused by the pathogen [25-34].

Although *E. coli* serotype O104:H4 has rarely been associated with human diseases in the past [35], outbreaksof infections caused by thisstrain was responsible for the clinical signs of HUS and bloody diarrhoea in humans in Germany, France, UK, Canada and the USA [8,9,22,36-42]. Moreover, epidemiological analysis involving pulsed-field gel electrophoresis, semi-automated rep-PCR and optical mapping analysis revealed that isolates from the different countries had similar genetic profiles [39]. It is therefore suggested that the isolates from Germany might have been transmitted through food sources, water or by person-to-person contact to the other countries.

To the best of our knowledge, there is currently no available information on the occurrence of *E. coli* O104 strains in humans, animals, environmental samples and food products in South Africa. Given the fact that *E. coli* O104:H4 has been associated with diseases in countries with more advanced public health care facilities there is a need to screen for the presence of these pathogens in South African food products. In this study we present the first investigation on the occurrence of enterohaemorrhagic *E. coli* O104 strains in raw meat products. This was designed to determine whether these meat products may serve as a potential source for the transmission of the organisms to humans.

2. Materials and Methods

2.1. Sample Collection

A total of 19 rawmeat samples including beef, mince, and lamb were purchased from butcheries in Mafikeng and local retail shops in Mabule and Legabane respectively. The samples were properly labeled and transported on ice to the laboratory for selective isolation of *E. coli*. Table 1 indicates the type and number of samples collected from the different areas.

Table 1.

Sample ID	Sample Station	Number of Samples Collected
MM1	Mafikeng	2
MB1	Mafikeng	2
MM2	Mafikeng	2
MM2	Mafikeng	2
MB3	Mafikeng	2
ML1	Mafikeng	1
ML2	Mafikeng	1
LM1	Legabane	2
MM1	Mabule	2
MB1	Mabule	2
LB1	Legabane	1
Total		**19**

2.2. Selective Isolation of *Escherichia coli* Species

Upon arrival in the laboratory, 2 grams of each meat sample was washed in 10mL of 2% (w/v) peptone water. Ten-fold serial dilutions were prepared using sterile 2% (w/v) peptone water. Aliquots of 100µL from each dilution was spread-plated on sorbitol MacConkey agar and used for the detection of lactose-fermenting (red colonies) and non-lactose fermenting (white colonies).Plates were incubated aerobically at 37°C for 24 hours. Sixteen presumptive colonies from each plate were purified by sub-culturing on sorbitol MacConkey agar and the plates were incubated aerobically at 37°C for 24 hours. These isolates were stored at room temperature and used for bacteria identification tests.

2.3. Determination of Cellular Morphology

The morphology of presumptive isolates was determined using standard methods [43]. Gram negativerod shaped bacteria were retained for further identification tests.

2.4. Triple Sugar Iron Test

Triple Sugar Iron agar (Biolab, Merck Diagnostics, South Africa) was used to differentiate members of the Enterobacteriaceae from other Gram-negative bacteria. The test was used to access the ability of isolates to breakdown the three sugars glucose, lactose and sucrose that are present at concentrations of 0.1%, 1.0% and 1.0%. Pure cultures were stab-inoculated into the butt and streaked on the surface of the slant of the TSI agar. The tubes were then incubated at 37°C for 24 hours. Results were read and isolates were classified based on the ability to ferment the sugars with or without the production of gas, colour change from red to yellow and the production of hydrogen sulphide (H_2S) [44].

2.5. Oxidase Test

This test was performed using the Test Oxidase reagent™ (PL. 390) as recommended by the manufacturer (Mast Diagnostics, Neston, and Wirral, UK). Positive results were recorded based on the production of a purple or blue colour and vice versa.

2.6. Simmons Citrate Utilization Test

Isolated pure colonies were streaked on the slant and a stab inoculated into the butt of Simmons Citrate agar (Fluka, Biochemika).The tubes were incubated at 37°C for 24 hours and observed for colour change from green to blue which was recorded aspositive results.

2.7. Sorbitol Fermentation Test

Isolates wereinoculatedinto a test tube containing phenol red sorbitol broth and incubated at 37°C for 24 hours. Positive results were based on a colour change from red to yellow [45].

2.8. Biochemical Characterization

The isolates were examined for biochemical properties of *E. coli* using the API 20E system (Bio-Mérieux, Marcy

l'Etoile, France) according to the manufacturer's instructions. The 20 biochemical tests performed included o-nitrophenyl-β-D-galactosidase, lysine decarboxylase, ornithine decarboxylase, urease production, citrate utilization, deamination of phenylalanine, malonate utilization, Esculin hydrolysis, fermentation of arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, sucrose, trehalose, raffinose and glucose; production of indole and acetoin and testing for the production of cytochrome oxidase. Results were read with or without the addition of reagents and the API web software was used to determine the identities of isolates based on the indices that were generated.

2.9. DNA Extraction

Genomic DNA was extracted from all presumptive isolates using a modified cell boiling method [46]. To carry out the procedure 500µl of sterile water was placed in 1.5 mL microfuge tubes and pure cultures of the isolates were transferred into the tubes. The tubes were vortexed vigorously to prepare a homogenous suspension. The cell suspension was incubated at 100°C in a heating block (Biorad, Digital dry bath) for 15 minutes and this was followed by centrifugation for 2 minutes at 13500 rpm. After centrifugation, the tube was placed on ice for 5 minutes and the supernatant was transferred to a new tube. An aliquot of 5µL of this supernatant was used for PCR analysis.

2.10. Identification of Suspected *E. coli* O104 Isolates by PCR Analysis

A PCR-based protocol was used to screen isolates for the presence of enterohaemorrhagic *E. coli* O104 strains through the amplification of the wzx_{O104} gene fragments [47]. Specific primer sequences O104F (TGTCGCGCAAAGAATTTCAAC) and O104R (AAAATCCTTTAAACTATACGCCC) [47] were utilized and amplifications were performed using a C1000 Touch ™ Thermal Cycler (Bio-Rad, Johannesburg, South Africa. Standard 25 µL PCR mixtures that contained 12.5µl master mix (Thermo scientific PMM), 8.5µL of nuclease free water, 1.5µL of loading buffer, 0.5µL oligonucleotide both primers and finally, 2µL of DNA template were prepared. All the reagents were Fermentas, USA products and were obtained from Inqaba Biotec Ltd, Sunnyside- South Africa. PCR amplification was performed according to the following in-laboratory optimized conditions: initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 64°C for 1 minute, extension at 72°C for 1 minute 30 seconds and final extension at 72°C for 5 minutes. The PCR products were stored at 4°C before separation by electrophoresis.

2.11. Electrophoresis of PCR Products

The PCR products were resolvedby electrophoresis on a 2% (w/v) agarose gel. A horizontal Pharmacia biotech equipment system (model Hoefer HE 99X; Amersham Pharmacia biotech, Sweden) was used to carry out electrophoresis and this was run for 2h at 80V using 1x TAE buffer (40mM Tris, 1mM EDTA and 20mM glacial acetic acid, PH 8.0). Each gel contained a 100bp DNA molecular weight marker (Fermentas, USA). The gels were stained in ethidium bromide (0.001µg/ml) for 15 minutes and the amplicons were visualized under U.V light at a wavelength of 420nm [48]. A Gene Genius Bio Imaging System (Syngene, Synoptics; UK) was used to capture the image using GeneSnap (version 6.00.22) software. GeneTools (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 *E. coli* O104:H4 Using Preliminary and Confirmation tests

A total of nineteen meat samples that comprised samples from beef, mince, and lamb were collected from four butcheries in Mafikeng, four shops in Mabule and three shops in Legabane. Sixteen characteristic isolates from each sample were subjected to bacteria identification tests for *E. coli* (Gram staining, oxidase test, Citrate utilisation test, TSI and API 20E test). Detailed results of the number of isolates that were positive for the different tests are shown in Table 2 and Table 3. All the isolates were Gram negative rods and therefore satisfied that presumptive characteristic for *E. coli*. A large proportion (93%) of these isolates was oxidase negative; fermented the sugars in the TSI medium; utilized citrate and fermented the carbohydrate sorbitol. On the contrary, only a small proportion (27.3%) of the isolates produced hydrogen sulphide gas (Table 2). Based on patterns obtained for the biochemical profiles of the isolates 102 (33.6%) were positively identified as *E. coli* (Table 3).

Table 2. Proportion of isolates that were positive for *E. coli* characteristics based on the preliminary biochemical tests

Sample site	GS	OT	SF	H_2S	Gas	CUT	SFT
	-ve	-ve		+ve		-ve	+ve
MWM 1	16	13	14	3	6	16	13
MWM 2	16	15	16	0	11	16	8
MBB 1	16	16	16	1	4	16	16
MBB 2	16	14	16	6	12	16	16
MBM 1	16	11	10	0	1	16	8
MBM 2	16	16	15	1	9	16	12
MM 1	16	15	16	8	15	16	15
MM 2	16	16	16	1	15	16	16
MB 1	16	16	15	13	16	16	15
MB 2	16	13	16	2	13	16	16
MLAB	16	13	14	0	6	16	13
MLBFF	16	15	16	13	12	16	9
LM 1	16	16	15	0	11	16	6
LM 2	16	16	16	11	9	16	7
MM 1	16	16	16	6	11	16	3
MM 2	16	16	16	16	16	16	3
MBV 1	16	16	15	0	11	16	13
MBV 2	16	16	16	2	7	16	14
LBV	16	16	16	0	14	16	14
Total	**304**	**285**	**290**	**83**	**199**	**304**	**217**

Table 3. Number of isolates that were positively identified as *E. coli* O104 based on specific PCR analysis

Sample site	No. screened by API 20E and PCR	No positive for *E. coli*	Proportion of *E. coli* O104 isolates that were positively identified from the different sample sites.
MWM 1	16	13	11
MWM 2	16	16	15
MBB 1	16	7	2
MBB 2	16	11	10
MBM 1	16	4	0
MBM 2	16	0	0
MM 1	16	9	0
MM 2	16	0	0
MB 1	16	0	0
MB 2	16	16	14
MLAB	16	8	0
MLBFF	16	0	0
LM 1	16	0	0
LM 2	16	7	0
MM 1	16	6	0
MM 2	16	2	0
MBV 1	16	3	0
MBV 2	16	0	0
LBV	16	0	0
Total	**304**	**102**	**52**

3.2. PCR for the Detection of *E. coli* O104:H4

A total of 304 *E. coli* isolates were screened for characters of enterohaemorrhagic *E. coli*O104 strains by specific PCR amplification of the wzx_{O104} genetic marker. Table 3 indicates the number of isolates that were positive for the target gene from the different sampling areas. Overall a total of 52 isolates were positively identified based on the presence of the wzx_{O104}. Despite this, not all samples were positive for this pathogen. However the detection of the recently emerged enteroaggregative *E. coli* O104 strain in raw meat samples in the North West Province, South Africa was a cause for concern. On the basis of the findings obtained in our study, *E. coli* O104 strains were isolated from raw meat samples and these food products may serve as potential vehicles for transmitting these pathogens to humans if the food products are consumed undercooked. Figure 1 indicates a 1% (w/v) agarose gel of the wzx_{O104} gene fragments amplified.

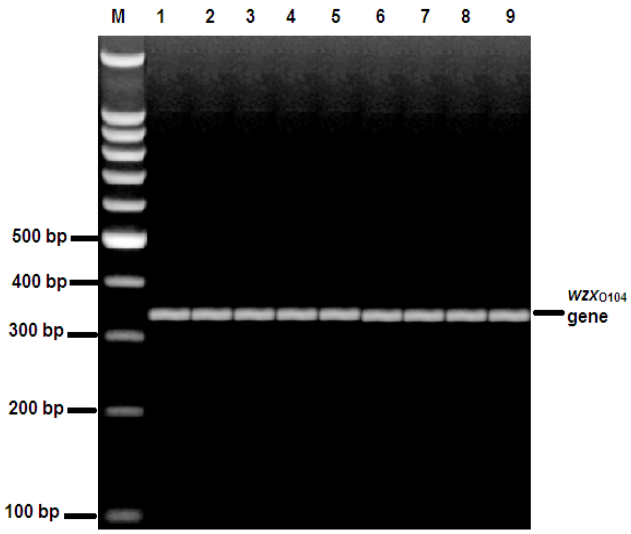

Figure 1. Agarose gel of wzx_{O104}gene fragments amplified from *E. coli*O104 isolates. Lane M= 100bp marker; Lanes 1-9= wzx_{O104} gene fragments amplified from *E. coli*O104 isolates obtained from meat samples

4. Discussions

It has been established that domestic and wildlife animals are the natural reservoirs of STEC and EHEC and the presence of these bacteria in the environment results through the uncontrolled release of faeces [15]. Therefore, these bacteria pathogens have the potential to contaminate food and water bodies if proper hygiene is not implemented. Bacterial foodborne zoonotic infections are the most common cause of human intestinal disease in many countries. This explains the need for enhanced research efforts and surveillance programs to enforceappropriate control measures by the food industry and also awareness to consumers [49]. Shiga toxin-producing *E. coli* are currently considered an important group of foodborne zoonotic pathogens due to the fact that they are frequently associated with diarrhoea, haemorrhagic colitis (HC) and the life threatening haemolytic uraemic syndrome (HUS) in humans in many countries worldwide [50]. Domestic animals especially pigs and cattle have been reported as the major reservoirs of STEC in South Africa [30,31,33] and other parts of the world [51]. There is currently no report describing the detection of non *E. coli* O157:H7 strains from food products in the North West Province of South Africa.

The objective of this study was to isolate *E. coli* O104 strains in meat samples collected from different supermarkets in the Mafikeng area and to identify these isolates using Gram staining, oxidase test, Triple Sugar Iron test, citrate utilization, Sorbitol fermentation tests, API 20E biochemical profiles and PCR analysis. In this study, *E. coli* O104 was detected and isolated from raw meat products that are sold in some supermarkets. The *E. coli* O104 strains were identified using both preliminary microbiology identification methods and a PCR assay. Most of the studies conducted on these species employed PCR assays only for their detection from samples [19]. Generally, there are two different approaches that are currently used to detect STEC in foods [52,53,54,55,56,57]. Serogroup-independent techniques rely on the prevalence of genes and the characteristics of contaminating STEC strains [52,54] while serogroup-dependent methods target a subset of *E. coli* serogroups that frequently implicated in human diseases and outbreaks and may be directed at *E. coli* O26, O45, O91, O103, O111, O121, O145 and O157 strains [53,55,56,57]. Consequently, other *E. coli* serotypes such as O113:H21, O174:H21 and the newly emerging O104:H4 serotype that are less frequently associated with human infections, but can still cause cases of HUS and can contaminate food products will therefore be missed [58]. In addition most of the protocols designed to detection of Shiga toxin-producing *Escherichia coli* (STEC) in food and water sources [27,28,29,30,32,59] typically focus on *E. coli* O157:H7 [27,28,29,32,59]. Against this background there is need to develop species specific assays to constantly and affectively monitor the occurrence of these non-O157 strains in food and water.

In France cattle was not a reservoir for the highly virulent *E. coli* O104:H4 strains [60]. However, other related *E. coli* O104 strains have been isolated from contaminated milk in the USA and sprouts in Germany [20]. EHEC strains are present in the gastrointestinal tract of domestic animals and during the removal of intestinal

contents, faecal contamination or contact between the hide and carcass can facilitate the transmission of pathogenic *E. coli* strains to the meat [15,61]. Moreover in most developing countries hygiene conditions are highly compromised [62] and therefore these increase the chances of contamination [16]. The implementation of routine pathogen surveillance strategies would great reduce human infections. This study reveals the presence of *E. coli* O104 strains in raw meat samples obtained from some supermarkets in the North West Province, South Africa. There is need to identify virulence determinants that would serve as definitive markers for determining the pathogenicity of the current strains.

5. Conclusion

In the present study *E. coli* O104 strains were successfully isolated from raw meat products and these isolates could have severe health complications on humans if thefood products arenot properly cooked before consumption. Given the complications posed by this pathogen in some European countries recently, coupled with the present baseline data it is suggested that a full scale study should be conducted to determine the occurrence of this organisms in a number of food products. An investigation of their virulence gene determinants and the genetic relationships of isolates from different sources will be of great epidemiological value.

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