Molecular identification of *Escherichia coli* O145:H28 from beef in the North West Province, South Africa

Josephine Chileshe and Collins Njie Ateba*

Department of Biological Sciences, School of Environmental and Health Sciences, Faculty of Agriculture Science and Technology, North-West University – Mafikeng Campus, P. Bag X2046 Mmabatho 2735, South Africa
atebacollins1@hotmail.com

Abstract: *Escherichia coli* occur as normal flora in the gastrointestinal tract of humans and animals. However, some strains belonging to the serotype O145:H28 and O104:H4 have been reported to cause life threatening infections in human worldwide with the most recent outbreak caused by the latter recorded in some European countries. The serotype *E. coli* O157:H7 has been extensively studied in the North West Province South Africa. However, there is currently no information on the occurrence of other enterohaemorrhagic *E. coli* strains. The aim of the study was to determine the occurrence of *E. coli* O145:H28 in meat samples using preliminary biochemical tests and PCR analysis. A total of nineteen meat samples were obtained from retail shops in Mafikeng, Mabule and Lagabane in the North West Province, South Africa. Sorbitol MacConkey agar was used for isolation of *E. coli* O145:H28 isolates. A total of three hundred and four (304) presumptive isolates were subjected to standard preliminary biochemical (Gram staining, oxidase test, TSI test and MUG test) tests. All the presumptive isolates from the three sampling stations were Gram negative rods. Large proportions of the isolates (92% and 90%) were oxidase positive and could not utilise citrate. Similarly, 82% of these isolates were able to produce gas from fermentation of the three sugars in the TSI medium while only 15% of them were capable of producing hydrogen sulphide. However, a large proportion (88%) of the isolates were able to ferment sorbitol. When subjected to *E. coli* O145:H28 specific PCR analysis, a large proportion (93%) of the isolates were positively identified. The number of *E. coli* O145:H28 isolates obtained was higher in samples obtained from Mafikeng (63.6%) than in Mabule (19.4 %) and Lebagane (17.0%). Based on the results from this study, the meat samples were contaminated with *E. coli* O145:H28. It is therefore recommended that meat products should be properly cooked before consumption to reduce human infections.

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1. Introduction

Escherichia coli belong to a large bacterial family, known as the *Enterobacteriaceae* (Donnenberg and Whittam, 2001). These are enteric bacteria, that are facultative anaerobic Gram-negative rods and they live in the gastrointestinal tracts of animals and humans (Donnenberg and Whittam, 2001). *Escherichia coli* are genetically heterogeneous and members of this genus are typically not considered as pathogens since they form part of the normal micro-flora of the intestinal tract of humans and animals (Karmali, 1989). However, strains within these species have acquired virulence genes determinants that enable them to cause intestinal or extra intestinal infections in their hosts (Kaper *et al*., 2004). Infections can range from uncomplicated diarrhoea to bloody diarrhoea and the more complication forms of diseases that include haemorrhagic colitis (HC), Haemolytic Uraermic Syndrome (HUS) and thrombotic thromcytopenic pupura (TTP). These diseases are known to account for a large proportion of renal failure in infected subjects and are also known to pose severe challenges to individuals who are immune-compromised (Momba *et al.,* 2008).

The *E. coli* that cause enteric disease have been divided into different pathotypes, based on their virulence factors and mechanisms through which they cause diseases in their hosts (Nataro and Kaper, 1998; Kaper *et al*., 2004). One of these patho-types, is known as Shiga toxin-producing *E. coli* and is abbreviated STEC. These are *E. coli* strains that produce at least one or more of potent cytotoxins that are called the shiga toxins. These toxins are so called because they are biologically and structurally similar to the toxins produced by *Shigella dysenteriae* (O'Brien *et al*., 1982). Moreover, they are also referred to as verotoxins due to the fact that they produced a profound and irreversible cytopathic effect on vero cells (Konowalchuk *et al.,* 1977).

Owing to the pathogenic properties of STEC strains they are also termed enterohaerrhagic. *E. coli* (EHEC) (Nataro and Kaper, 1998). The STEC comprises more than 400 different *E. coli* serotypes and not all STEC strains potentially pathogenic (Karmali *et al*., 2003; Scheuts and Strockbine, 2005). However, EHEC serotypes such as O26:H11,

O103:H2, O111:H8, O118:H16, O121:H19, O145:H28 and O157:H7 have been found to cause disease in some parts of the world (Bugarel *et al.,* 2010; Centres for Disease Control and Prevention, 2009; European Food Safety Authority (Tzschoppe *et al,* 2012; EFSA, 2009; Karmaili *et al.,* 2003; Maidhof *et al.,* 2002; EFSA working group, 2002; Tarr *et al*., 2002; Mead *et al*., 1999). It is therefore suggested that the occurrence and the pathogenicity of these strains could only be determined after surveillance studies and laboratory analysis. Although the occurrence may also vary from region to region, it is important to mention that only the prevalence of *E. coli* O157:H7 in animals and humans is known in the area (Ateba and Mbewe, 2011; Ateba *et al*., 2008; Ateba and Bezuidenhout, 2008).

Considering the fact that an EHEC strain belong to the serotype O104:H4 was implicated as the cause of diarrhoeal infections in European countries that have more advanced public health facilities, it is therefore important to investigate the occurrence of these strains in South African food products.

2. Materials and methods

2.1 AREA OF STUDY

This research was conducted in Mafikeng in the North-West Province, South Africa. Meat samples were collected from various supermarket and butcheries located within the Mafikeng area.

2.2 METHODS

2.2.1 Sample collection and analysis

Nineteen meat samples were purchased from various randomly selected supermarkets and butcheries in the Mafikeng locality. These samples were placed in plastic sample collection bags and transported to the laboratory for analysis on ice. On arrival in the laboratory, immediately approximately 5g of each meat sample was washed in 5ml of 2% buffered peptone water (Biolab, Merck Diagnostic, South Africa). Aliquots of 100µl from each dilution were plated onto sorbitol-MacConkey agar (SMAC) supplemented with cefixime (50ng/ml) and potassium tellurite (25mg/ml).The plates were incubated at 37°C for 24hours (Meichtri *et al*., 2004).

2.2.2 Gram Staining

Gram staining is an experimental method used for differentiating bacteria species based on the chemical and physical properties of their cell walls (Gram-negative and Gram-positive).Presumptive isolates on sorbital MacConkey agar were subjected to the Gram satin reaction using standard methods(Cruikshank *et al*., 1975). An isolated pure colony was placed on the microscopic slide and a drop of sterile water was added and mixed to make a smear which was fixed by passing of the slide through the flame. The cells were stained with crystal violet dye (primary stain) for a minute and the excess stain was gently with tape water. This was discoursed with an iodine solution (mordant). The cells were decolorized with 70% alcohol (ethanol). Finally the cells were counterstained with Safranin O for a minute, washed with tape water gently and then air dried. The morphology of the cells was determined by examining the slide under oil immersion on a light microscope.

2.2.3 Preliminary biochemical identification tests

2.2.3.1 Oxidase Test

This test was performed using the Test Oxidase reagent™ (PL.390) as recommended by the manufacturer, Mast Diagnostics, Neston, and Wirral, U.K. The oxidase is based on the principle that tetramethly-p-phenylenediamine is oxidized by cytochrome in the presence of atmospheric oxygen to form purple coloured compound .In carrying out the test; a pure isolated colony was picked using a sterile wire loop and placed on a Whatmans filter paper. A drop of the Oxidase™ reagent was added to make a smear. After 30 seconds the formation of a purple or dark blue compound was the indication that the isolate is oxidase positive and if no colour change indicated that the isolate was oxidase negative.

2.2.3.2 Triple Sugar Iron Test (TSI)

An isolated pure colony was sub inoculated into the butt and streaked on the surface of the slant on the TSI agar by use of a sterile wire loop (Biolab, Merck Diagnostic, South Africa). The agar was incubated at 37°C for 24 hours. The results were recorded based on fermentation of the three sugars (Lactose, Sucrose and Glucose) and this is indicated by the change of medium colour of the butt and slant to red or yellow, production of hydrogen sulphate gas and gas production (Forbes and Weissfeld, 1998).

2.2.3.3 Simmons Citrate Utilization Test

An isolated pure colony was streaked on the slant of the Simmons Citrate agar (Fluka, Biochemika) that was contained in 10 ml McCartney bottles by use of a sterile wire loop. The media were inoculated at 37°C for 24hours. After inoculation the positive results were recorded based on the medium colour change from green to blue while the negative results were recorded based on no colour change.

2.2.3.4 Sorbitol fermentation Test

The test was performed to determine the ability of the isolates to ferment carbohydrate sorbitol as a carbon source. In performing the test 1% sorbitol was prepared in nutrient broth and phenol red indicator was added. An isolated pure colony was transferred to a sterile tube 5ml of the broth and incubated at 37°C for 24 hours. The tubes were assessed visually for colour change from pink to yellow. The results were recorded and interpreted as previously described (Kiiyukia, 2003).

2.2.4 Confirmatory biochemical identification

2.2.4.1 Extraction of genomic DNA

Genomic DNA was isolated from pure isolates by the boiling method (Tunung *et al*., 2007). Pure cultures were revived by spread-plating onto nutrient agar plates and the plates were incubated aerobically at 37°C for 24 hours. After incubation, 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 votexed vigorously to prepare homogenous suspensions. The cell suspensions were 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 tubes were placed on ice for 5 minutes and the supernatant was transferred to a new tube. Aliquots of 5µl of the supernatants were used for PCR analysis. All the isolates were subjected to PCR analysis for identification as *E. coli* O145:H28 using specific primers that are shown in the Table 3.1.

2.2.5 Identification of suspected *E. coli* O145:H28 isolates by PCR

The suspected *E. coli* O145:H28 will be confirmed through the amplification of the *E. coli* $ihp1_{0145}$ and $fliC_{H28}$ gene fragments using specific primer sequences (Table 1).

Table 1. The suspected *E. coli* O145:H28 will be confirmed through the amplification of the *E. coli* $ihp1_{0145}$ and $fliC_{H28}$ gene fragments using specific primer sequences.

Primers	Target gene	Sequence (5′→3′)	Product size (bp)
O145F	$ihp1_{O145}$	CGATAATATTTACCCCACCAGTACAG	370
O145R		GCCGCCGCAATGCTT	
H28F	$fliC_{H28}$	AAAACAATGCTGGGACTGTC	620
H28R		TTGTAATTACCGTAGATACGGC	

2.2.6 Electrophoresis of PCR products

The PCR products were separated by electrophoresis on a 2% (w/v) agarose gel, for target genes. 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 (Fermentars, 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 (Sambrook *et al.,* 1989). 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

3.1 Detection of *E. coli* O145:H28isolates in meat samples using preliminary biochemical tests

A total of nineteen meat samples were analysed for the presence of *E. coli* using preliminary biochemical tests. Only isolates that satisfied the preliminary characteristics for *E. coli* were used for molecular identification tests. A total of 304 presumptive isolates were screened for characters of *E. coli* and all of the isolates (100%) were Gram negative rods that were also able to ferment the three sugars glucose, lactose and sucrose in the TSI medium. Large proportions of the isolates (92% and 90%) were oxidase positive and could not utilise citrate. Similarly, 82% of these isolates were able to produce gas from fermentation of the three sugars in the TSI medium while only 15% of them were capable of producing hydrogen sulphide. However, a large proportion (88%) of the isolates were able to ferment sorbitol. The results showed that isolates that satisfied the preliminary characteristics for *E. coli* were frequently isolated from samples obtained in Mafikeng than Mabule and Labagane. The results in Table 2 indicate the number of isolates screened from the different sampling areas and those that were positive for the different tests. All the isolates that satisfied the presumptive characteristics for *E. coli* were subjected to confirmatory identification *E. coli* O145:H28 using molecular methods.

Table 2: Results of preliminary tests for *E. coli* isolated from meat samples

Sample Station	NT	Oxidase Test		SCT		TSI			SOF
		+	-ve	+ve	-ve	SF	H_2S	Gas	+ve
Mafikeng	192	10	182	5	187	192	19	150	172
Mabule	64	8	56	15	49	64	5	60	52
Legabane	48	6	42	10	38	48	23	40	45
Total	**304**	**24**	**280**	**30**	**274**	**304**	**47**	**250**	**269**

3.2 PCR for the identification of *E. coli* O145:H28 isolates using $ihp1_{O145}$ and $fliC_{H28}$ primers

All of the 305 isolates that were screened using the preliminary identification tests for *E. coli* were subjected to specific PCR analysis to confirm their identities as *E. coli* O145:H28. This was achieved through amplification of the $ihp1_{O145}$ and $fliC_{H28}$ gene fragments that are specific for *E. coli* O145:H28 isolates. A large proportion (93%) of the isolates were positively identified and results are shown in Table 4.2. Figure 4.2 indicates a 2% (w/v) agarose gel showing the $ihp1_{O145}$ gene fragments that were amplified. As shown in Table 4.2, the number of *E. coli* O145:H28 isolates obtained were higher in samples obtained from Mafikeng (63.6%) than in Mabule (19.4 %) and Lebagane (17.0%).

Table 3: Number of *E. coli* O145:H28 isolates positive for the targeted genes

Sample source	No of isolates tested	No of isolates positive for the target genes	
		$ihp1_{O145}$	$fliC_{H28}$
Mafikeng	192	180	180
Mabule	64	55	55
Lebagane	48	48	48
Total	**304**	**283**	**283**

4. Discussion and Conclusions

The main objective of this study was to isolate and identify *E. coli* O145:H28 from meat samples obtained from retail shops in North West Province. *E. coli* stains have been identified as causative agents of life threatening diseases such as HC and HUS in humans (Karmali *et al*., 2003; Nataro and Kaper, 1998). A motivation is the fact that enterohaemorrhagic *E. coli* strains have recently been implicated as the cause of gastrointestinal disease in humans in some European countries (Tzschoppe *et al,* 2012). Despite this, there is no information on the occurrence of these strains in South African food products. Moreover, the shiga toxin producing *E. coli* O157:H7 strain has been extensively studied in the area and both genotypic and phenotypic tools have been used to optimise protocols that could be used for source tracking (Ateba and Mbewe, 2011; Ateba *et al*., 2008; Ateba and Bezuidenhout, 2008). It is therefore important to determine the occurrence, biochemical characteristics and the virulence gene determinants in other pathogenic *E. coli* stains.

In the present study almost all the samples were positive for *E. coli* O145:H28. Proportion isolates obtained were higher in samples obtained from Mafikeng (63.6%) than in Mabule (19.4 %) and Lebagane (17.0%). This may have resulted from the fact that in Mafikeng the shops recieve very high numbers of customers when compared to the other two stations sampled. Therefore, the fact that shop attendants may have to serve too many persons at the same time may lead to improper hygiene practices. Moreover, these microbes may have originated from the GIT tract of farms animals and contaminated the environment used for slaughtering, machines used cutting the meat. It has been demonstrated that these type of contaminats are easily transferred to meat and the level of contamination is found to increase if the personnel working in both the processing plants and sale points do not practice proper hygiene (Yilmaz *et al*., 2006; Tutenel *et al*., 2003; Bouvet *et al*., 2001).
Based on results presented herein, we suggest that the prevalence of *E. coli* O145:H28 in meat sold in the supermarkets largely depends on hygiene conditions in abattoirs, the surrounding environments and among the personnel that handle the meat (Ateba and Mbewe, 2011).

In conclusion the detection of *E. coli* O145:H28 in beef is a cause for concern and this may indicate the burden of foodborne diseases that may occur on consumers in the area. Despite the fact that there is country to country and region to region variation in foodborne diseases attributable to beef, there are three general factors that largely contribute to the recorded incidences (Rhoades *et al.,* 2009). These include the pathogen load in beef products that are consumed; the per-capita consumption of beef products in the country consumed and the cooking and consumption habits of the country (Rhoades *et al.,* 2009). Although, there is no quantitative information on the consumption of raw and lightly cooked meat products in South Africa and the North West province in particular, it is suggested that there is the need to properly cook these meat products before they are consumed. This therefore indicates the need to strictly implement baseline and performance standard surveys on chilled beef carcasses in abattoirs in the North West province. Moreover, for public health and safety reasons, it is important to improve hygiene practices at the farms in which the animals are kept.

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Correspondence to:
Dr. C.N Ateba
Department of Biological Sciences, School of Environmental and Health Sciences, Faculty of Agriculture Science and Technology, North-West University – Mafikeng Campus, P. Bag X2046 Mmabatho 2735, South Africa.
Telephone: +27-78-334-4878;
Emails: atebacollins1@hotmail.com

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