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### Prevalence and antibiogram profiles of *Escherichia coli* O157:H7 isolates recovered from three selected dairy farms in the Eastern Cape Province, South Africa

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#### ABSTRACT

**Objective:** To investigate the occurrence and antibiotics susceptibility of *Escherichia coli* (*E. coli*) O157:H7 isolates from raw milk, cattle udder, milking machines and worker's hand swabs from three selected commercial dairy farms in the Amathole District Municipality, Eastern Cape Province, South Africa.

**Methods:** Raw milk samples were collected from bulk storage tanks and swab samples were collected from milking machines, cattle udders and worker's hands fortnightly over a six-month sampling regime between June and November 2014. A standard culture-based method was used for the enumeration and isolation of *E. coli* O157:H7, presumptive identification using sorbitol MacConkey agar (supplemented with cefixime (50 µg/L) and potassium tellurite (25 mg/L). A serological confirmation of the presumptive *E. coli* O157:H7 isolates was conducted using the O157 latex agglutination test kit.

**Results:** A total of 252 *E. coli* O157:H7 isolates were further subjected to PCR amplification of *rfbE*<sub>O157</sub> and *fliC*<sub>H7</sub> genes of which 27(11%) of the isolates were confirmed positive *E. coli* O157:H7. The percentage antibiotic resistance of the 27 *E. coli* O157:H7 isolates from the dairy farms revealed penicillin [23 (85%)], tetracycline [22 (81%)], erythromycin [19 (70%)], streptomycin [14 (52%)] and chloramphenicol [12 (45%)]. The highest resistances were penicillin [23 (85%)] and tetracycline [22 (81%)].

**Conclusions:** These findings revealed that the dairy farms are potential reservoirs of *E. coli* O157:H7 serotype, and harbor antibiotic-resistant determinants, a concern to public and environmental health.

## 1. Introduction

The emergence of *Escherichia coli* (*E. coli*) O157:H7 serotype dates back to 1982 when it was first discovered in an outbreak traced to contaminated Hamburgers[1]. Ever since its discovery to date *E. coli* O157:H7 remains as one of the most imperious foodborne pathogens, known to cause bloody diarrhoea,

haemolytic uremic syndrome and hemorrhagic colitis in humans almost everywhere in the world[2].

Antimicrobial resistance has developed as an alarming health concern over time[3]. The Enterobacteriaceae, such as *E. coli* (with its variants) and some *Klebsiella* spp., produces different β-lactamase enzymes, some of which have activities against penicillin as well as second and third generations of cephalosporins. However, they have been reported to have improved their β-lactamases activity in recent years with the capability to hydrolyze the extended spectrum cephalosporin which led to the rapid evolution of extended spectrum β-lactamases with a capacity to confer resistance towards β-lactamase and non-penicillin antibiotics[4-6]. It is quite apparent that resistant bacteria evolves naturally when these bacterial strains self-replicate spontaneously or horizontally

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through genetic transfer mechanisms by microorganisms with resistant characteristics in conjunction with those that do not[7]. The multi-drug resistant isolates, particularly *E. coli*, have shown an alarming increase and wide resistance capability to broad-spectrum antimicrobials which are consequent causes of treatment failures, resulting to high mortality rates[6,8].

To facilitate and enhance the production among dairy farms, it has been conventional practices that most farmers tend to use antibiotics as growth promoters which may have a different interaction in the animal somehow enabling the spread and development of antibiotic resistance to some bacterial population[9]. Agricultural practices and the abnormal use of antimicrobials in veterinary medicine often promote the antimicrobial resistant bacteria and their positive selective pressure[3]. Inadequate clinical waste treatment may contribute in the prevalence and persistence of antimicrobial resistant bacteria and antibiotic residues in the environment, which becomes a major concern to global communities[10].

A number of studies suggest that the exploitative use of antimicrobial agents in humans and animals may support the increased resistance patterns by *E. coli* strains including O157:H7 to antimicrobials[11]. Antimicrobial resistance is reported as a massive setback towards effective prevention and treatment of the ever-increasing infections by bacteria, parasites, fungi and viruses, which is a global threat and a worrisome concern to the world of medicine[12]. The use of antibiotics for growth promotion and antimicrobial agents in dairy farms solely as treatment regime against *E. coli* is common in the Eastern Cape Province with the ever-increasing development of antimicrobial resistance by this bacterium[11]. To the best of our knowledge, there is scarcity of information on the prevalence and antibiogram characterization of *E. coli* O157:H7 in dairy farm surroundings in the Eastern Cape Province, South Africa. Hence, the present study elucidates the prevalence and antimicrobial susceptibility profiles of the confirmed *E. coli* O157:H7 isolates from three selected dairy farms in the Eastern Cape Province of South Africa.

## 2. Materials and methods

### 2.1. Description of the study location

Three selected commercial dairy farms under the Amathole District Municipality in the Eastern Cape Province, South Africa were used for this study and for confidentiality purpose were identified as farms A, B and C, respectively. Dairy farm A was surrounded by a number of villages and peri-urban settlements. It was located on the geographical coordinates 32°37'0" S and 27°07'0" E. This dairy covers about 700 hectares of land with

about 400 cows, a production capacity of 2000 L of milk per day with 36 workers. Dairy farm B was located on the geographical coordinates of 32°49'0" S and 26°59'0" E and covered a terrain of about 280 hectares of land with 600 cows producing 2000 L of milk per day with 16 permanent workers. Dairy farm C was situated along the geographical coordinates of 32°47'0" S, 26°50'0" E. About 800 cows were milked daily in the farm which produced an estimate of 10000 L milk per day and had a total of 10 full-time workers. It supported both the local region and other regions abroad the Amathole District Municipality borders with its produce.

### 2.2. Sample collection

Samples were collected forth nightly over a period of six months (June–November, 2014). Samples included raw bovine milk samples from farm bulk storage tanks and were collected using pre-sterilized 50 mL centrifuge tubes (3 tubes for each farm), while sterile swabs sticks (Copan Group, Copan, Italia) were used to collect samples from milking machines, udder and hands of workers, and all samples were appropriately labelled. Samples were then transported on ice pack to the Applied and Environmental Microbiology Research Group Laboratory at the University of Fort Hare and analysed within few hours of collection.

### 2.3. Isolation and identification of *E. coli* O157:H7

Isolation of *E. coli* O157:H7 from raw milk samples was carried out following the protocol as described by Ateba and Mbewe[13] with some modifications. For raw milk samples, tenfold dilutions ( $10^{-1}$ – $10^{-3}$ ) of milk were made using sterile physiological buffer saline (PBS), where 1 mL of raw milk sample was transferred into 9 mL of sterile PBS ( $10^{-1}$  first dilution) and another 1 mL from the  $10^{-1}$  dilution was transferred into another 9 mL of sterile PBS, and the process was repeated until  $10^{-4}$  dilution was reached. One hundred microliter from each dilution was immediately spread-plated (in triplicates) on sorbitol MacConkey agar (Laboratorios Conda, Pronadisa, South Africa) plates supplemented with cefixime (50 µg/L) and potassium tellurite (25 mg/L) (Oxoid culture media supplements, UK) for the detection of *E. coli* O157:H7 and then incubated at 37 °C overnight. Colonies that appeared colourless or exhibited a beige colour on the agar were considered as presumptive *E. coli* O157:H7 positive isolates.

Swab samples from milking machines, cattle udders and the hands of workers collected across the three farms were inoculated into 10 mL of trypticasein soy broth (Laboratorios Conda, Pronadisa, South Africa) and incubated on a shaker at 37 °C overnight at 150 r/min. At the end of the incubation period,

100 µL of each turbid culture was sub-cultured on sorbitol MacConkey agar plates supplemented with cefixime (50 µg/L) and potassium tellurite (25 mg/L) (Oxoid culture media supplements, UK) using spread plate technique and incubated at 37 °C overnight. Colourless or beige colonies were picked as presumptive *E. coli* O157:H7 isolates. The presumptive *E. coli* O157:H7 isolates were then purified by repeated aseptic transfer onto fresh nutrient agar plates to obtain pure isolates and stored on sterile 25% glycerol stock. Serological test for *E. coli* O157:H7 was conducted for further confirmation of the presumptive *E. coli* O157:H7 isolates using the latex agglutination test (*E. coli* O157 Latex Test Kit, Oxoid, UK), following the manufacturer's instructions.

#### 2.4. DNA extraction

DNA extraction from the *E. coli* O157:H7 isolates was conducted using the boiling method as described by Gugliandolo *et al.*[14]. Briefly, pure isolates were inoculated into sterile trypticasein soy broth (Laboratorios Conda, Pronadisa, South Africa) and incubated at 37 °C overnight. At the end of the incubation, 2 mL from the previously grown culture was transferred into sterile 2 mL Eppendorf tubes (Biologix Research Co., Lenexa, KS) and centrifuged at 11 000 r/min for 10 min, the obtained pellet was washed twice using sterile distilled water before re-suspending into 200 µL of sterile distilled water. The suspension was boiled at 100 °C for 10 min in a heating block (Lasec, UK). The boiled cell lysate was immediately cooled at -20 °C for 10 min, followed by centrifugation at 12 000 r/min for 5 min. The supernatant was then carefully transferred into new sterile micro-centrifuge tubes and used as template DNA for PCR amplification. *E. coli* O157:H7 ATCC 35150 was used as reference strain.

#### 2.5. PCR amplification of *E. coli* O157:H7

PCR amplification was performed in a 25 µL reaction mixture in a 200 µL tube with 12.5 µL of master mix (Thermo Scientific, USA), 0.25 µL two specific genes *rfbE* and *fliCH7* (Table 1) each of forward and reverse primers (Integrated DNA Technologies, USA), 2 µL of nuclease free water (Thermo Scientific, USA) and 10 µL of template DNA. The amplification was performed using thermal cycler (Bio-Rad, Mycycler™ thermal cycler, South Africa). Primer pairs used in the amplification were listed in Table 1. The thermal conditions for the PCR were as follows: initial denaturation at 95 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 90 s, extension at 72 °C for 90 s, and final extension at 72 °C for 5 min and the amplicons were held at 4 °C[17]. The PCR products were then subjected to gel

electrophoresis using 2% (w/v) agarose (Separations, South Africa) gel with 0.5 Tris-borate-EDTA buffer at 100 V for 60 min. The gel was stained with 5 µL ethidium bromide and 100 base-pair ladder (Thermo Scientific, USA) was used as the DNA size marker and the gel was visualized under UV transilluminator (Alliance 4.7, UVITEC, UK).

**Table 1**

Primer sequences and expected size of PCR amplified genes targeted in the isolates.

Primer	Primer sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
<i>FliCH7</i>	TACCATCGCAAAGCAACTCC GTCGGCAACGTTAGTGATACC	<i>fliC<sub>H7</sub></i>	247	[15]
<i>RfbE</i>	CTACAGGTGAAGGTGGAATGG ATTCTCTCTTTCCTCTGCGG	<i>rfbE<sub>O157</sub></i>	327	[16]

#### 2.6. Antimicrobial susceptibility testing

The antimicrobial susceptibility test was performed on Mueller-Hinton agar (Laboratorios Conda, Pronadisa, South Africa) plates using the standard disc diffusion method of Kirby-Bauer recommended by the Clinical and Laboratory Standards Institute[18]. Briefly, fresh *E. coli* O157:H7 isolates from sorbitol MacConkey agar plates were sub-cultured on nutrient agar (Merck, South Africa) and incubated at 37 °C for 18–24 h. After incubation, a loopful of colonies was inoculated on physiological buffer saline to make up a bacterial suspension adjusted to 0.5 McFarland standards. A sterile swab stick was then deepened into the prepared bacterial suspension and spread evenly on the entire surface of Mueller-Hinton agar (Laboratorios Conda, Pronadisa, South Africa) plates and allowed to stand for about 15 min. Thereafter, different antibiotic discs (Mast Diagnostics, UK) [amikacin (30 µg), streptomycin (10 µg), gentamycin (10 µg), tetracycline (30 µg), doxycycline (30 µg), oxytetracycline (30 µg), cephalothin (30 µg), cefotaxime (30 µg), cefoperazone (75 µg), chloramphenicol (30 µg), ampicillin (10 µg), penicillin G (10 µg), polymyxin B (300 units), erythromycin (15 µg), sulfamethazole/trimethoprim (25 µg), trimethoprim (25 µg), sulfamethaxazole (25 µg)] were placed equidistance on the lawn of bacteria using antibiotic disc dispenser (Mast Diagnostics, UK) and the plates were incubated at 37 °C for 18–24 h. After incubation, the plates were examined for zones of inhibition and interpreted based on the interpretation standard of the Clinical and Laboratory Standard Institute[18]. These antibiotics were frequently used in the treatment of *E. coli* O157:H7-related illnesses, thus they were selected for this study design.

### 3. Results

A total of 252 presumptive *E. coli* O157:H7 isolates were subjected to serological tests for the presence of the O antigen

using the O157 latex agglutination test kit. Out of these, 27 (11%) were positive for the O antigen suggesting to be *E. coli* O157:H7. The 27 isolates were further confirmed by PCR technique using two sets of primers, *rfbE* and *flicH7* (Table 1) which targeted the *RfbE*<sub>O157</sub> and *FliC*<sub>H7</sub> genes, respectively. PCR products were then subjected to 2% agarose gel for electrophoresis which was observed at 327 and 247 base pairs, respectively.

The highest incidence of *E. coli* O157:H7 was obtained from cattle udders in Farm A with 55% of the presumptive isolates positive for the strain. Indeed, the frequency of detection of the organism was generally high at this farm and ranged between 33.3% and 54.5%. In Farm B, the frequency of detection ranged from 5.9% to 17.2%, while in Farm C, the frequency of detection varied from 0.0% to 13.3%.

Only 27 (11%) isolates were confirmed as positive *E. coli* O157:H7 and these were further subjected for their antimicrobial susceptibility profiles. The results showed multidrug resistance against penicillin [23 (85%)], tetracycline [22 (81%)], erythromycin [19 (70%)], streptomycin [14 (52%)] and chloramphenicol [12 (45%)]. The highest resistances were obtained against penicillin [23 (85%)] and tetracycline [22 (81%)] (Table 2).

**Table 2**

Antimicrobial susceptibility patterns of *E. coli* O157:H7 isolates collected from the three dairy farms ( $n = 27$ ).

Antibiotic class	Antimicrobial agent	Percentage resistant profile ( $n = 27$ )		
		S [ $n$ (%)]	I [ $n$ (%)]	R [ $n$ (%)]
Aminoglycosides	Amikacin (30 µg)	19 (70)	5 (19)	3 (11)
	Streptomycin (10 µg)	12 (44)	1 (4)	14 (52)
	Gentamycin (10 µg)	13 (48)	5 (19)	9 (33)
	Tetracycline (30 µg)	5 (19)	0 (0)	22 (81)
Tetracyclines	Doxycycline (30 µg)	18 (66)	5 (19)	4 (15)
	Oxytetracycline (30 µg)	14 (52)	7 (26)	6 (22)
	Cephalothin (30 µg)	17 (63)	6 (22)	4 (15)
Cephalosporins	Cefotaxime (30 µg)	20 (74)	1 (4)	6 (22)
	Cefoperazone (75 µg)	16 (59)	5 (19)	6 (22)
Phenicol	Chloramphenicol (30 µg)	9 (33)	6 (22)	12 (45)
Penicillins	Ampicillin (10 µg)	3 (12)	12 (44)	12 (44)
	Penicillin G (10 µg)	0 (0)	4 (15)	23 (85)
Polymoxin	Polymoxin B (300 units)	14 (51)	5 (19)	8 (30)
Macrolide	Erythromycin (15 µg)	8 (30)	0 (0)	19 (70)
	Sulfamethazole/trimethoprim (25 µg)	21 (77)	1 (4)	5 (19)
	Trimethoprim (25 µg)	19 (70)	3 (11)	5 (19)
Folate pathway inhibitor	Sulfamethaxazole (25 µg)	5 (19)	2 (7)	20 (74)

R: Resistant; I: Intermediate; S: Susceptible.

#### 4. Discussion

Disease associated with the pathogenic *E. coli* O157:H7 serotype has been reported almost everywhere in the world with a very few unreported cases[19]. The present study was aimed at assessing the prevalence of *E. coli* O157:H7 in raw milk, milking machines, cattle udders and worker's hand swabs collected from three selected dairy farms in the Eastern Cape Province, South

Africa. A total of 252 presumptive *E. coli* O157:H7 isolates were obtained from these farms, but only 27 (11%) isolates were confirmed as *E. coli* O157:H7 using two different primers sets *RfbE* and *FlicH7*. Previous studies have reported low prevalence of *E. coli* O157:H7 in raw milk from storage bulk tanks[20]. However, when taking into careful consideration, the low infection dose of this pathogen (about 100–200 or even less than 10 cells in susceptible consumers) is still a major public health-risk concern[21,22]. The significance of *E. coli* O157:H7 pathogen from others is characterised by the ability to infect individuals at very low infectious doses. Their unusual acid tolerance and their association with animals are mostly primary food sources to humans[23].

It was observed that the cattle udders were mostly covered in faeces and there was no form of sterilization carried out prior to milking, hence contamination of milking machines and consequently raw milk was probably as a result of the dirty udders and the milking machine rotary system. In the present study, 5 (19%) isolates obtained from raw milk, another 5 (19%) from milking machines and 2 (7%) from milk handler's or workers' hands were compared to 15 (55%) which were isolated from cattle udders through the six-month sampling regime, these findings suggest that cross-contamination may be possible during the milking process. Several authors have also highlighted that there are various factors that contribute greatly in milk contamination in dairy industries and these factors include poor hygienic milking conditions, contaminated equipments, milking utensils and milk handlers' poor personal hygiene[17]. That is in line with the findings of the present study as isolates were recovered from the milking utensils, suggesting poor hygienic practises. In a similar study by Caine *et al.*[24], 54% prevalence of *E. coli* O157:H7 isolates was reported from raw cattle milk samples collected from selected Eastern Cape commercial dairy farms, signifying cattle as important carrier for this pathogen. A certain portion of people from rural areas still consume unpasteurised milk either directly or indirectly through milk products[25]. Since milk supports a wide range of microbial growth[26,27], pasteurisation is thus an important alternative in the control of milk-borne pathogens that threaten public health[28].

Antimicrobial resistance among enteric bacteria has become a global burden over the past years, playing a fundamental role in restricting treatment options in sickness control and treatment therapy with evidence of transmission of resistant pathogenic strains to humans through food[9,29]. *E. coli* O157:H7 isolates showed multidrug resistance with penicillin, tetracycline, erythromycin, streptomycin and chloramphenicol in the present study. The maximum reaction percentages of 81% and 85% towards tetracycline and penicillin were observed, respectively. A study of the same nature by Reuben *et al.*[9] obtained similar

patterns of resistance to penicillin and tetracycline with the latter being commonly used as first line drug by humans, growth promoter and routine chemoprophylaxis by various farmers among their livestock in Nigeria, which may be an explanation to the high resistance level of this antibiotic. In a similar study by Iweriebor *et al.*[11] in the Eastern Cape Province, South Africa, their findings deduced that *E. coli* O157:H7 isolates from cattle faeces exhibited multidrug resistance. This is a troublesome finding with regards to public health and human safety.

The information gathered from the three dairy farms which used both penicillin and tetracycline excessively resulting from the high resistances obtained in our study suggests an urgent intervention to improve general well-being and diminish public health risks. According to Popowska *et al.*[3], most antibiotics are partially degraded in waste treatment plants with tetracycline and erythromycin not degraded, which suggests the accessibility of such residues in the environment and consequently high resistances were revealed. However, with the isolates susceptible to some of the antibiotics in the following proportions: amikacin [19 (70%)], doxycycline [18 (66%)], cefotaxime [20 (74%)] and gentamycin [13 (48%)] (Table 2). A similar study conducted by Iweriebor *et al.*[11] reported high prevalence of multidrug resistance to various antimicrobial agents among 95 *E. coli* O157:H7 isolates obtained from dairy cattle faeces in the Eastern Cape Province, South Africa. According to their report, resistance was observed in the following magnitude: tetracycline (97%), oxytetracycline (95%), ampicillin (95%), cephalothin (95%), chloramphenicol (90%), streptomycin (84%), trimethoprim/sulfamethazole (84%) and cefuroxime (82%). A phenomenon referred to as horizontal gene transfer could be responsible for dispensing resistance genes to susceptible bacteria via bacterial plasmid, which may elevate the tenacity of antimicrobial resistant bacteria in the environment and thus a great public health risk factor[6,7,29].

There is a lack of substantive treatment for *E. coli* O157:H7 infection and acute diarrheal illnesses have been proven antibiotics ineffective and some reports suggest that antimicrobials somehow increase haemolytic uremic syndrome development by the release of enterotoxins by damaged bacteria[12]. One of the limitations in the incidences of *E. coli* O157:H7 is that infected individuals tend to not seek medical attention or health care especially those in rural areas, which results in undetected or rather unknown or ignored cases. Generally, personal hygiene, consuming of pasteurized milk and milk products, sufficiently cooked foods including clean-fresh fruits and vegetables and use of sufficiently treated water for any purpose can play a vital role in preventing enterohaemorrhagic *E. coli* infections[21,30-32]. The three studied dairy farms supported both rural and peri-urban communities in the Eastern Cape

region, and remained as one of the major affiliates towards the province's economic growth and social welfare. However, it is very troubling to enumerate such disturbing findings revealed in the present study which evidently highlights the amount of public and environmental health risk that the province is confronted with at the moment. Although the prevalence and antimicrobial profiles of *E. coli* O157:H7 isolates have been thoroughly investigated in the present study, yet a future research can be designed towards elucidating the antimicrobial resistance gene determinants in order to detect and establish the presence and distribution of virulence/resistance genetic marker(s) in the phenotypically resistant *E. coli* O157:H7 strains.

The findings of this research have established that three dairy farms in the Eastern Cape Province, South Africa are potential reservoirs of pathogenic and antibiotics-resistant *E. coli* O157:H7 serotype in its environments. These dairy farms provide enormous support to the rural and peri-urban populaces of the Amathole region. However, these findings indicate a worrisome concern with regards to public and environmental health, and we suggest the necessity of public health intervention measures, effective communication and education advocacy to the communities. Finally, the use of antibiotics in the dairy farms should be controlled to prevent the frequency of multi-antibiotic resistant *E. coli* O157:H7 strains.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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