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# Molecular Detection of *Clostridium* Species in Beef Obtained from Retail Shops in North West Province, South Africa

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**Abstract** *Clostridium* species cause diseases in humans that result from consumption of undercooked beef. The objectives of this study were to isolate *Clostridium* species from beef then the detection of the presence of *tpi* housekeeping gene as well as determination of the antibiotic resistant profile of the isolates. Twenty six (26) beef samples were bought from butcheries, supermarkets and street vendors. The samples were analyzed for the characteristics of *Clostridium* species and a total of 78 presumptive isolates were subjected to Gram-staining, catalase test, API 20A sugar fermentation profiles 16S rRNA and *tpi* species specific PCR analysis. Susceptibility profiles to 8 antibiotics were determined and antibiotic resistance patterns were compiled. Large proportions (93.3%-100%) of the isolates were penicillin, vancomycin and erythromycin resistant. PCR were performed to amplify species-specific 16S rRNA gene to confirm the identity of the isolates and 44.7% of the isolates were positively identified as *Clostridium* species. PCR were performed to amplify *tpi* housekeeping gene fragments. The *tpi* housekeeping gene produced amplicons of 501bp after PCR amplification and 19% of the isolates possess *tpi* housekeeping gene which confirmed the presence of *Clostridium* species in beef.

***Keywords:*** *Clostridium species, tpi housekeeping gene, beef, PCR analysis*

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## 1. Introduction

*Clostridium* species are Gram-positive, rod shaped, anaerobic and obligate bacteria that are capable of forming endospores and thus giving them the ability to endure hostile environmental conditions [1,2,3]. Clostridia can exist as free-living bacteria or as pathogens that infect both humans and animals [4]. The genus *Clostridium* contains 181 identified species that are grouped into 19 clusters [4,5]. Cluster I comprises mostly of *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens* and *Clostridium tetani* that are known to cause severe diseases in humans and therefore have huge clinical significance [2,3,4,6,7].

Virulent strains of *C. difficile* have also been reported to cause food-borne and nosocomial outbreaks of infections in humans and these usually present fatal colitis, enteritis and diarrhea even in countries in North America and Europe that have access to proper public health and health care facilities [8,9,10,11,12]. In 2003, it was reported that *C. difficile* was responsible for the death of more than 2000 people in Quebec, Canada [13] Moreover, recent studies conducted in Europe have also shown that the mean incidence of complications caused by *Clostridium* species has risen tremendously from 2.45 cases per 10,000 patients that visit the hospital for diarrheal related infections in 2005 to 4.1 cases per 10,000 patients in 2008 [14,15]. This therefore indicates that *Clostridium* species are a severe problem even in countries with advanced public health facilities.

Despite the fact that animals are known to be the primary reservoirs for *Clostridium* species, a number of food products have been reported to transmit these pathogens to humans if proper hygiene is not practiced [12,13,14,15,16]. Infections caused by *Clostridium* species usually result from the ingestion of food contaminated with clostridia endospores [12]. The endospores are capable of developing into vegetative cells within the gastrointestinal tract of the host and the latter produces potent toxins that have cytopathic effects on the epithelial cells.

Under unfavorable conditions, *Clostridium* species form spores which survive both standard cooking and food processing measures [4] To date, there are a number of food products in which *Clostridium* has been isolated, and these include ground meat, raw and ready-to-eat meat, salads, and even water [17] Similarly, *Clostridium difficile* was isolated from ground beef and ground pork with ground beef obtaining the highest percentage (71%) [18] Moreover, large proportion (66%) of C. *perfringens* was

isolated from ground meat [19]. Despite all these, *Clostridium* species are seldom considered to be zoonotic [3]. However, transmission of *Clostridium* species may occur via direct contact with host animals or through the consumption of contaminated food of animal origin [20,21]. Unlike other *Clostridium* species, *Clostridium difficile* is transmitted mostly through fecal-oral routes [8,22,23].

*Clostridium* species can infect a host but without any clinical signs and symptoms [7]. Despite this in some patients, symptoms may range from uncomplicated watery diarrhea, to bloody diarrhea and life threatening complications such as stiff and flaccid muscles [3,13,24]. In the North West Province of South Africa, there is currently no information on the occurrence of *Clostridium* species in food products. The present study is aimed at determining the occurrence of these pathogens in retail meat and street vended beef meals. Data obtained will serve as baseline information for further studies and also provide opportunities for surveillance mechanisms to be put in place.

# 2. Materials and Methods

## 2.1. Meat Sampling

A total of 26 meat (beef) samples were purchased from butcheries supermarkets and street vendors in the North West Province during the months of March to July 2013. Samples were obtained from rural and urban areas Table 1. The samples were properly labeled and transported on ice to the Molecular Microbiology Research Laboratory in the Department of Biological Sciences for analysis. Upon arrival, the samples were analyzed immediately for the presence of *Clostridium* species, but where analysis was not possible due to time constraints; the samples were stored in a refrigerator at 4$^{o}$C and were analyzed within 24 hours.

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

| Sampling area | Number of samples |
|---|---|
| Dingateng | 1 |
| Mafikeng | 10 |
| Logagane | 1 |
| Lichtenburg | 3 |
| Mabule | 2 |
| Potchefstroom | 3 |
| Carltonville | 3 |
| Coligny | 2 |
| Ventersdorp | 1 |

## 2.2. Isolation of the Bacteria

Approximately, 5g of each meat sample from both raw and cooked vendors was cut aseptically with a sterile forceps and the samples were washed or vortexed vigorously in 10ml of 2% peptone water (Biolab, S.A.). Ten-fold serial dilutions were prepared and aliquots of 100µl from each dilution were spread-plated on Brain Heart Infusion agar that was supplemented with 5% bovine blood. The plates were incubated at 37°C for 3 days under microaerophilic (10% $CO_2$) conditions [25]. After incubation, presumptive *Clostridium* isolates were sub-cultured on Brain Heart Infusion agar and the plates were incubated at 37°C for 3 days under microaerophilic (10% $CO_2$) conditions [25]. Isolated colonies were subjected to specific identification tests that facilitate the detection of *Clostridium* species.

## 2.3. Bacterial Identification

Isolates were identified using the following criteria:

### 2.3.1. Preliminary Identification

#### 2.3.1.1. Cellular Morphology

The morphology of presumptive *Clostridium* isolates was determined by Gram-staining using standard techniques [26]. All isolates that were Gram-positive rods were subjected to the catalase test [27,28].

#### 2.3.1.2. Catalase Test

The catalase test is commonly used to determine the presence of the catalase enzyme which degrades toxic hydrogen peroxide ($H_2O_2$) in cells containing the cytochrome oxidase system. A pure colony was transferred onto the surface of a microscopic slide using a sterile inoculating needle. A drop of 3% hydrogen peroxide was added and the slide was observed for the presence of bubbles. The results were recorded in the data sheet and all isolates that were catalase negative were presumptively considered to be *Clostridium* species.

### 2.3.2. Confirmatory Biochemical Tests

#### 2.3.2.1. Analytical Profile Index (API) 20A

Twenty representative isolates were randomly selected and subjected to API 20A test for identification as members belonging to the genus *Clostridium*. The test was performed according the manufacturer's instructions (BioMérieux, Marcy-L'Etoile / France). Briefly, fresh colonies from Brain Heart Infusion agar were used to make bacterial suspensions. The suspensions were mixed with API 20A medium provided. The microtubules were inoculated with the suspensions as instructed. The strips were placed into trays which were hydrated with 5ml distilled water to create humid atmosphere. The strips were incubated using anaerobic incubator for 24 hours. Results were read with or without the addition of reagents. The indices were generated for the different isolates and these were used to determine their identities using the API web $^{TM}$ identification software.

#### 2.3.2.2. Molecular Characterisation of *Clostridium* Species

**DNA extraction**

Pure isolates from the Brain Heart Infusion agar were inoculated into 5ml of nutrient broth and incubated anaerobically at 37°C for 24 hours while shaking. Genomic DNA was extracted from all presumptive *Clostridium* isolates using Zymo Research Genomic DNA$^{TM}$ –Tissue MiniPrep kit (Catalog No. D3050 & D3051-USA supplied by Biolab, South Africa) following instructions from the manufacturer. Briefly, 100µl from the broth culture was aliquoted into 1.5 mL sterile eppendorf tube, then 95µl 2X digestion buffer and 5µl of Proteinase K were added. The contents of the tube were vortexed and incubated at 55$^{o}$C for 20 minutes using a

pre-heated Bio-Rad heating block (Digital dry heat- Bio-Rad). After 20 minutes, 700µl of Genomic Lysis Buffer was added to the tubes and the contents were vortexed vigorously to facilitate the lysing of the cells. The mixture was transferred to a Zymo-Spin™ IIC Column in a collection tube. The contents of the tube were centrifuged at 13500 rpm using a bench-top Hermle Z300 high speed microcentrifuge (Kendro, Germany) for 1 minute and the supernatant in collecting tube was discarded. The pellet was re-suspended in 200µl of DNA Pre-buffer to each spin column in a new collection tube, centrifuged as mentioned above and the resulting supernatant was discarded. About 400µl of g-DNA Wash Buffer was added to the spin column and centrifuged at 13500 rpm for 1 minute and the resulting supernatant was discarded. Finally, 100µl of DNA Elution Buffer was added to each tube, the tubes were incubated for 5 minutes and then centrifuged at 13500 rpm for 30 seconds to elute the DNA into the new eppendorf tube. The DNA concentration was determined by using NanoDrop 2000 Spectrophotometer (Thermo Scientific) with a wavelength of 260 nm. DNA samples were stored at -20°C for future use.

#### 2.3.2.2.1. Amplification of *Clostridium* Species-specific 16S rRNA Gene

*Clostridium* species-specific 16SrRNA gene target sequences were amplified from all isolates using the primer sequences [29]. The primer pair 16S rRNAF (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 16S rRNAR (GTGGACTACCAGGGTATCTAATCC-3′) were used to amplify an 800 bp gene fragment. Amplification was performed using a previously published protocol but with minor modifications [29]. Amplifications were carried out using a C1000 Touch ™ Thermal Cycler (Bio-Rad, Johannesburg, South Africa. PCR amplification was performed in a total volume of 25µl made up of 12.5µl master mix (Thermo scientific PMM), 8.5µl of nuclease free water, 1.5µl of loading buffer, 0.5µl oligonucleotide primer set and finally, 2µl of DNA template. All the reagents were obtained from Inqaba Biotec Ltd, Sunnyside- South Africa. PCR amplification was performed according to the following parameters: initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°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.

#### 2.3.2.2.2. Detection of *tpi* Housekeeping Gene

The *tpi* gene for identification of *Clostridium* species was performed using specific oligonucleiotide primer combinations [29]. The primers tipF (5′-GCW GGW AAY TGG AAR ATG MAY AA-3′) and tipR (5′-TTW CCW GTW CCD ATW GCC CADAT-3′) were used in the assay. PCR amplification was performed using DNA thermal cycler (model - Bio-RAD C1000 Touch™ Thermal Cycler). PCR amplification was performed in a 25µl reaction mixture consisting of 12.5µl master mix (Thermo scientific PMM), 8.5µl of nuclease free water, and 1.5µl of loading buffer, 0.5µl oligonucleotide primer set and 2µl of DNA template. All PRC reagents were Fermentas USA products obtained from Inqaba Biotec Ltd, Sunnyside-South Africa. PCR amplifications were performed according to the following parameters: pre-denaturation at 94°C for 2 minutes, followed by 46 cycles of denaturation at 94°C for 30 seconds, annealing at 41°C for 1minute, extension at 72°C for 2 minute and a final extension at 72°C for 5 minutes. The PCR products were stored at4°C for future use.

### 2.4. Electrophoresis of PCR Products

The PCR products were separated by electrophoresis on a 2% (w/v) agarose gel. A horizontal Pharmacia biotech equipment system (model-BCMSCHOICE; Biocom, UK) was used to carry out electrophoresis and the gel was run for 90 minutes at 80V and 250MA using 1x TAE buffer (40mM Tris, 1mM EDTA and 40mM glacial acetic acid, PH 8.0). Each gel contained a 100bp DNA molecular weight marker (Fermentas, USA). The gel was stained in ethidium bromide (0.1µg/ml) and amplicons were visualized under U.V light at 420nm wavelength [30]. A ChemiDoc Imaging System (Bio-Rad ChemiDoc™ MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software.

### 2.5 Antibiotic Susceptibility Test

Antibiotic susceptibility test was performed using the Kirby-Bauer disc diffusion method [31]. A pure colony of *Clostridium* isolate from a fresh culture was used to prepare a bacterial suspension. Aliquots of 100µl from the suspensions were spread-plated on Mueller Hinton agar (MH) using a sterile cotton swab. Antibiotics discs were place on the inoculated MH agar plates and the plates were incubated under micro-aerophilic conditions for 24 hours. The susceptibilities of the isolates to a panel of eight different antimicrobial agents obtained from Mast Diagnostics, South Africa was determined. The following antimicrobial agents were used; Penicillin G (10 µg/ml), Amoxicillin (10 µg /ml), Erythromycin (15 µg /ml), Tetracycline (10 µg /ml), Vancomycin (30 µg /ml), Norflaxacin (10 µg /ml), Streptomycin (300 µg /ml) and Chloramphenicol (30 µg /ml). The test was performed and results interpreted according to the guidelines of the Clinical Laboratory Standards Institute [32]. Zones of inhibition around the discs were measured in millimeters and the standard reference values were used to classify isolates as being susceptible, intermediate resistant or resistant to a particular antibiotic [32].

## 3. Results and Interpretation

### 3.1. Screening of Clostridium Isolates Using Preliminary and Confirmatory Biochemical Test

A total number of twenty six (26) beef samples were analysed for the presence of *Clostridium* species using Brain Heart Infusion agar. Only those isolates that satisfied the preliminary (gram-staining catalase test,) confirmatory biochemical (API 20A and PCR analysis) for *Clostridium* species were retained for antibiotic susceptibility tests. A total number of seventy-eight (78) isolates were screened for characteristics of *Clostridium* species using Gram stain procedure and 75 of these were rods and Gram positive rods (Table 2). Additionally,

98.7% of these isolates tested negative for catalase test. Nevertheless, of the twenty isolates which were subjected to API 20A test only 15% isolates were positively identified as *Clostridium botulinum, Clostridium sordeli and Clostridium histolyticum*. A large proportion, 75% of these isolates were able to degrade urea and 65% did not produce indole. While all (100%) of the isolates were able to hydrolyze glucose, maltose and lactose. All the isolates that were positively identified as *Clostridium* species by preliminary and biochemical tests were subjected to confirmatory identification using molecular methods.

## 3.2 Molecular Characterisation of *Clostridium* Isolates from Beef

### 3.2.1. Analysis of DNA Quality

Genomic DNA was extracted using Zymo Research Genomic DNA™ –Tissue MiniPrep kit using the protocol as described in Materials and Methods (Section 3.4.). The DNA was resolved by agarose gel electrophoresis. Figure 1 shows an agarose (2% w/v) gel depicting genomic DNA extracted from the isolates. The DNA was of good quality and without fragmentation.

### 3.2.2 PCR for the Detection of Specific 16S rRNA Gene

Forty seven isolates were selected randomly, and subjected to *Clostridium* PCR analysis. Figure 1 indicates a 2 % (w/v) agarose gel depicting 16S rRNA gene fragments. The desired 800 base pairs fragments were obtained after running the gel electrophoresis for 90 minutes. The number of isolates that were positive for the 16S rRNA gene is shown in Table 3 and the data showed that 44.7% of *Clostridium* species were positively identified by specific PCR analysis. Nevertheless, a large proportion of positive isolates (47.62%) were detected in samples from Mafikeng area than were from Potchefstroom (14.29%).

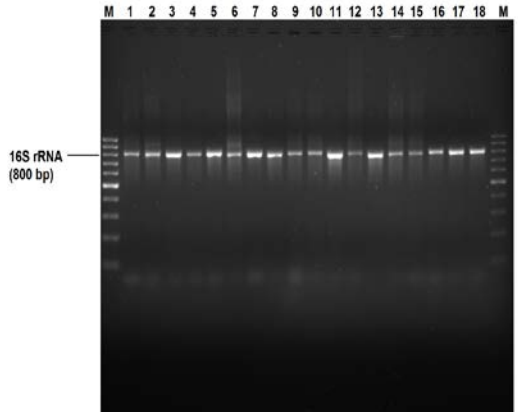


**Figure 1.** PCR detection of 16S rRNA gene. Lane M= 100 bp marker; lane 1-7 (*16S rRNA* gene fragments from DNA extracted from *Clostridium* isolated from beef- Mafikeng); lane 8 (*16S rRNA* gene fragment from DNA extracted from *Clostridium* isolated from beef- Mabule); lane 9-10 (*16S rRNA* gene fragments from DNA extracted from *Clostridium* isolated from beef- Lichtenburg); lane 10-12 (*16S rRNA* gene fragments from DNA extracted from *Clostridium* isolated from beef- Carltonville); lane 13-15 (*16S rRNA* gene fragments from DNA extracted from the *Clostridium* isolated from beef- Coligny) and lane 16-18 (*16S rRNA* gene fragments from a DNA extracted from *Clostridium* isolated from beef-Potchefstroom

**Table 2. Isolates that were positive for test used to confirm the identities of the bacteria strains in the study**

| Area | S ID | GS | CM | ST | CT | IT | UT | G,M,L |
|---|---|---|---|---|---|---|---|---|
| **Carletonville** | Cv3B | + | Rod | + | - | + | - | + |
| | CV3A | + | Rod | + | - | + | - | + |
| | CV2C | + | Rod | + | + | + | + | + |
| | CV1B | + | Rod | + | - | + | + | + |
| | CV1C | + | Rod | + | - | - | - | + |
| **Mafikeng** | MFK1A | + | Rod | + | - | + | + | + |
| | MFK3A | + | Rod | + | - | + | + | + |
| | MFK3B | + | Rod | + | - | + | + | + |
| | MFK1C | + | Rod | + | - | + | + | + |
| **Potchefstroom** | P3C | + | Rod | + | - | - | + | + |
| | P3A | + | Rod | + | - | + | + | + |
| | P2A | + | Rod | + | - | - | + | + |
| | P2C | + | Rod | + | - | + | + | + |
| | P3B | + | Rod | + | - | - | + | + |
| | P3C | + | Rod | + | - | - | + | + |
| **Lichtenburg** | L2A | + | Rod | + | - | + | + | + |
| | L2B | + | Rod | + | - | + | + | + |
| **Ventersdorp** | **V1C** | + | Rod | + | - | - | + | + |
| **Coligny** | Cg4B | + | Rod | + | - | - | - | + |
| | Cg4C | + | Rod | + | - | - | - | + |

SID = Sample Identity; GS=Gram staining status; CM=Cellular morphology; ST=Spore test; CT=Citrate Utilization; IT=Indole test; UT=Urease test; G, M, L= Glucose, maltose and lactose fermentation test

**Table 3. No of isolates that were positive for the 16S rRNA gene fragment PCR analysis**

| Area | No of isolates tested | No of isolates positive for the *16S rRNA* gene |
|---|---|---|
| **Carletonville** | 5 | 2 |
| **Coligny** | 8 | 3 |
| **Dingateng** | 3 | 0 |
| **Logageng** | 6 | 0 |
| **Lichtenburg** | 4 | 2 |
| **Mabule** | 2 | 1 |
| **Mafikeng** | 12 | 10 |
| **Potchefstroom** | 6 | 3 |
| **Ventersdorp** | 1 | 0 |
| **Total** | 47 | 21 |

### 3.2.3. PCR for the Detection of *tpi* Housekeeping Gene for *Clostridium* Species

Forty seven isolates were subjected to PCR analysis for the detection of *tpi* gene was determined using specific primer combinations for the targeted gene. The Figure 2 indicates a 2% (w/v) agarose gel depicting *tpi* gene fragments. The desired 501 bp fragments were obtained after running the gel electrophoresis for 90 minutes. The number of positive isolates from different stations shown in Table 4 and the data showed that nine (9) of *Clostridium* species were positively identified by *tpi* specific PCR analysis.

**Table 4. Results for specific *tpi* housekeeping gene fragment PCR analysis**

| Area | No of isolates tested | No of isolates positive for the *tpi* housekeeping gene |
|---|---|---|
| **Carletonville** | 5 | 0 |
| **Coligny** | 8 | 3 |
| **Dingateng** | 3 | 0 |
| **Logageng** | 6 | 0 |
| **Lichtenburg** | 4 | 1 |
| **Mabule** | 2 | 0 |
| **Mafikeng** | 12 | 4 |
| **Potchefstroom** | 6 | 1 |
| **Ventersdorp** | 1 | 0 |
| **Total** | 47 | 9 |

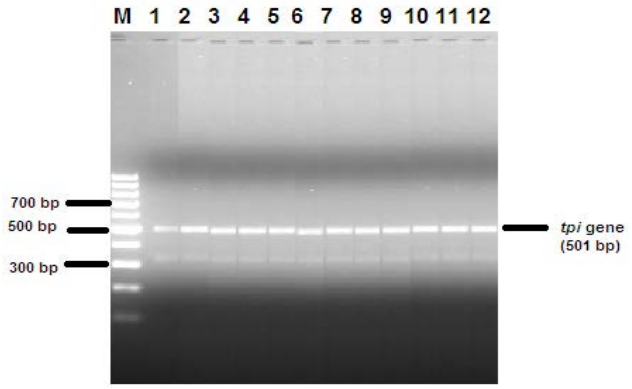


**Figure 2.** PCR detection of *Clostridium* species housekeeping *tpi* gene. Lane M= 100 bp marker; lanes 1-4= *tpi* gene from isolates in Mafikeng; lanes 5-7= *tpi* gene from isolates in Potchefstroom; lanes 8-10 *tpi* gene from isolates in Coligny and lanes 11-12= *tpi* gene from isolates in Lichtenburg.

## 3.3 Antibiotic Resistant Data of the Isolates

Antibiotic susceptibility profiles of *Clostridium* species isolated was tested against eight different antimicrobial agents and results were reported as percentages. Seventy four (74) isolates were tested to evaluate their resistance patterns. The resistance patterns obtained for the isolates tested are shown in Figure 3. As indicated in Figure 4, a large proportion (93.3%-100%) of the isolates from Mafikeng, Mabule, Carltonville, Potchefstroom and Ventersdorp were resistant to penicillin, vancomycin, and erythromycin. Despite this none of the isolates from Dingateng and Logagane were resistant to amoxicillin, streptomycin and norfloxacin. Similarly, none of the isolates from Mabule were resistant to streptomycin and norfloxacin. Moreover, none of the isolates from Dingateng and Lichtenburg were resistant to chloramphenicol. Despite the fact that none of the isolates from Dingateng were resistant to amoxicillin, chloramphenicol, norfloxacin and streptomycin, a small proportion (33.3%) were resistant to erythromycin, penicillin, tetracycline and vancomycin. However, a large proportion, 83.3%-100% of isolates from Mabule were resistant to amoxicillin, erythromycin, penicillin and vancomycin.

Interestingly, all the isolates from Mafikeng, Ventersdorp and Potchefstroom were resistant to all of the antibiotics tested. However, a large proportion, 60%-100% were resistant to amoxicillin, erythromycin, tetracycline and vancomycin. Despite this, a small proportion (16.7%) of the isolates form Potchefstroom were also resistant to amoxicillin. The data presented here indicated that multiple antibiotic resistant strains were obtained and it is therefore suggested that these isolates could serve as reservoirs for the transmission of antibiotic resistance genes within bacterial species and the human population.

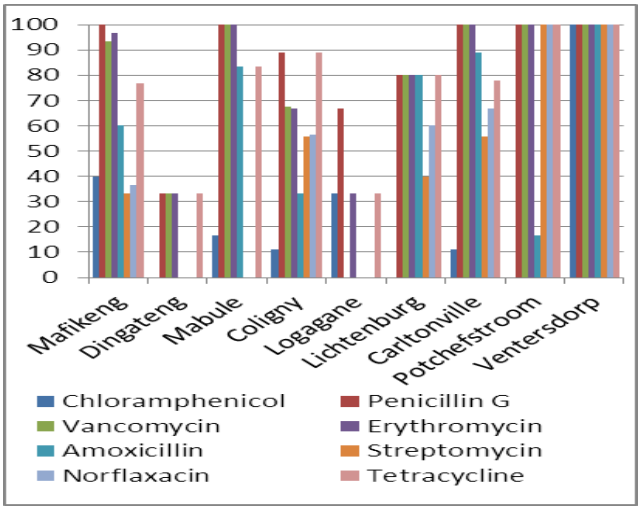


**Figure 3.** Antibiotic resistance patterns of the *Clostridium* isolates from different stations

# 4. Discussions

The primary objective of the study was to isolate and identify *Clostridium* species from raw beef purchased from retrial shops and butcheries in the North West Province, South Africa. A motivation for this was that food illness caused by *Clostridium* species is among the common illness resulting from consumption of contaminated beef. Contaminated food (beef) is considered as a vehicle for transmission of *Clostridium* diseases in human [33]. Transmission of *Clostridium* diseases via consumption of contaminated beef has been reported in developed countries [15,20,34]. *Clostridium* species cause sporadic cases of human diarrhoea, pseudomembranecolitis, tetanus, gangrene and botulism [4,8,9,10]. In a recent study conducted in Belgium, *Clostridium difficile* was mostly isolated from the intestines (9.9%) of cattle at the slaughter [35]. The prevalence *of Clostridium difficile* in cattle just before slaughter was ranging between 6.3-12% [36,37,38]. Contamination of meat can occur during evisceration process [39]. Therefore, this implies that poor sanitation and improper hygiene may exacerbate the transmission of *Clostridium* diseases to humans.

In rural communities, there is a high risk of acquiring *Clostridium* diseases owing to improper hygiene practices. Another problem which exists in rural communities is lack of storage facilities (refrigerator) to store meat either before cooking or after cooking [3,40]. Despite this, there is no information on the isolation of *Clostridium* species from raw beef in the North West Province, South Africa.

This study has successfully isolated *Clostridium* species from beef and the results obtained in this study concur with the published data [10,17,18,21,41,42]. In a study conducted in USA, the prevalence of *Clostridium* species in beef, pork and ground beef was reported to be 42.4%,

41.3% and 44.4%, respectively [41]. Nevertheless, the results obtained in this study indicate low percentages as compared to previous studies. The reason can be due to differences in the methods used and the source and type of samples.

A further objective of the study was to use species-specific 16S PCR analysis to confirm the isolates. The analysis of the 16S rDNA gene represents molecular approach for bacterial identification [29]. Moreover, this gene has been widely studied for bacteria identification at genus and or species level owing to its universal distribution. The rDNA gene is the most conserved DNA in almost all the cells, and the portion of rRNA sequence from distantly related organisms are remarkably similar [39]. This implies that sequences from distantly related organisms can be precisely aligned and make the true differences easy to measure [39,43]. Therefore, the results presented herein for 16S PCR amplification coincide with previous studies [29,43,44,45]

Another objective of the study was to evaluate the presence of *tpi* housekeeping gene in *Clostridium* species isolated from beef in the North West Province, South Africa. A motivation for this was the fact that such a study has not been conducted in this area and there is scarce data on isolation of *Clostridium* species from beef in South Africa, particularly in the North West Province. The triose phosphate isomerase (*tpi*) housekeeping gene encodes for a triose phosphate isomerase enzyme. Additionally, housekeeping enzymes are constitutively expressed in all organisms to perform essential metabolic functions for the purpose of their survival [46]. In case of pathogens, certain enzymes play a role to enhance virulence and to perform such a function, the enzyme must be located on the surface [46].

The *tpi* gene has been widely used for identification of *Clostridium* species. This gene was found to be more discriminatory than 16S rRNA gene for identification of *Clostridium* species [29,47]. Similarly, this can also provide an alternative marker to 16S rDNA for phylogenic analysis [29]. PCR-restriction analysis of the *tpi* gene offers an accurate tool for specific identification within the genus *Clostridium* [29,46,48]. This study successfully determined the presence of *tpi* housekeeping for *Clostridium* species using species-specific PCR and Figure 3 indicate fragments of the *tpi* housekeeping gene. This validates the data obtained from biochemical and 16S rRNA gene PCR analysis. These findings are in line with previous studies [29,37,46,47,48]. Despite this, the findings for this study showed a lower percentage of the isolates which possess *tpi* housekeeping gene.

Antibiotic resistance in bacterial isolates is a worldwide phenomenon and is a serious problem especially in developing countries [39]. Antimicrobials are used for prevention and or treatment of the diseases in humans and constant use of those antibiotics may lead to acquisition of microbial resistance and thus cause a threat to public health. Therefore, this pose a need to determine the level of antibiotic resistance among bacterial isolates [39]. Another objective of this study was to determine the extent to which *Clostridium* isolates from beef were resistant to antibiotics. A motivation for this was the fact that there is a concern of the possible development of resistance to antimicrobial agents in *Clostridium* species. *Clostridium* species especially C. *difficile* are most often associated with diarrhoea and are increasingly regarded as highly prevalent nosocomial pathogens that affect surgical and gastroenterologic patients and the elderly [49,50,51]. These bacteria species produce protein toxins, A (*TcdA*) and B (*TcdB*), that are known to be the main virulence factors [52] as well as a binary toxin (CDT) [53]. *C.* species and *C. difficile* are the most commonly identified pathogen of antibiotic-associated diarrhoea (AAD) in humans, but unfortunately in many cases they remain undiagnosed. *Clostridium perfringens* on the other hand is widely associated with hospital-acquired diarrhoea in humans [54,55]. This pathogen is known to even produce a *C. perfringens* enterotoxin in patients with AAD [56] whose level of production may increase during the sporulation phase that takes place in the gastrointestinal tract [57]. AAD most frequently caused by *C. difficile* is an increasing problem, leading to prolonged hospital stay and additional cost [53]. Given the fact that *Clostridium* species are responsible for approximately 20% of all antibiotic-associated diarrhoea cases in humans [51] it is very important to evaluate the occurrence of these pathogens in raw and street-vended food products. Moreover, an investigation of the antibiotic resistance profiles of isolates may provide an indication of the degree of health associated complications that the isolates in a given area may pose to humans.

This study reports the antibiotic resistance profile of *Clostridium* species isolated from beef. All 74 isolates tested were resistant to at least one of the antibiotic used in the study. Penicillin, vancomycin, erythromycin and tetracycline were four antibiotics to which a large number of isolates were resistant. Moreover, the isolates that were resistant to penicillin, vancomycin and erythromycin were frequently identified. Chloramphenicol was the drug to which most of the isolates were susceptible and this coincides with the previous studies [58,59,60]. However, a large number of isolates were resistant to penicillin, vancomycin, erythromycin, amoxicillin and tetracycline. In a previous study, many isolates were resistant to penicillin and amoxicillin [59], and this study represents similar findings.

## 5. Conclusion and Recommendations

The main objective of this study was to isolate *Clostridium* species from beef samples bought from supermarkets, retail shops and butcheries. A further objective was to confirm the identities of the isolates using PCR analysis as well as determine their antibiotic resistance profile. Isolates were successfully identified by based on the presence of *tpi* gene which is specific to *Clostridium* species. *Clostridium* species displayed high level of resistance to most of the antibiotics utilized. Isolates were most often resistant to penicillin, vancomycin and erythromycin. Despite the fact there was low resistance to chloramphenicol, all the isolates were resistant to at least one of the antibiotics tested. *Clostridium* species are responsible for approximately 20% of all antibiotic-associated diarrhoea cases in humans. This suggests that there is a need to implement surveillance programs that monitor the prevalence and antibiotic resistance among *Clostridium* species. This could ensure proper public health and thus contributing in

reducing the occurrence of *Clostridium* infections. Since the resistance profile of *Clostridium* species is unknown in this area, further studies must be conducted to determine the antibiotic resistant genes in *Clostridium* species. Such a study would provide more data concerning the antibiotic resistance of *Clostridium* species in the North West Province.

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