Isolation of vancomycin resistant enterococci isolated from leafy vegetables (lettuce) from North West Province

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Abstract: Seventeen leafy vegetable samples were collected from shops in Mafikeng. These samples were analysed for the presence of *Enterococcus* species. A total of 136 potential isolates were obtained based on colonial appearance and all the isolates were subjected to Gram staining, oxidase and catalase tests. Generally, all the isolates satisfied the preliminary identification tests for enterococci and their identities were confirmed using the 16S rRNA specific PCR analysis. A large proportion (60.3%) of enterococci was isolated from spinach when compared to lettuce (39.7%). Seventy eight isolates were positively identified as enterococci based on the PCR assay. The isolates were tested to determine their antibiotic resistant profiles against eleven different antibiotics. Generally a large proportion (72.1% to 100%) of the isolates were resistant to the antibiotics; amoxicillin, ampicillin, vancomycin, chloramphenicol, teicoplanin and erythromycin. On the contrary only small proportions (9% to 28.1%) of these isolates were resistant to tetracycline and doxycycline. Tetracycline and doxycycline are used to treat a number of infections and therefore the resistance pattern observed could not be explained. Similarly small proportions (27.3% to 37.5%) of these isolates were also resistant to ciprofloxacin. A course for concern is the fact that isolates that harbor multiple antibiotic resistant phenotypes including resistance to the drug vancomycin was detected during the study. These isolates were observed in lettuce that is consumed raw in the form of salad in many homes and ready to eat food outlets. It is therefore important to wash these food products properly before they are consumed.

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1. Introduction

Enterococci are Gram-positive, round shaped bacteria that live as normal flora in the gastrointestinal tracts of warm blooded mammals and are released to the environment through human and animal dejections (Busani *et al.,* 2004). However, some species within the genus have been found to cause diseases in their hosts and enterococci are therefore regarded as human pathogens (Patel, 2003). Infections caused by these organisms in humans include endocarditis, sepsis, bacteraemia, peritonitis and urinary tract infections (Foulquie-Moreno, 2006).

The treatment of enterococcal infections, particularly endocarditis, requires the use of aminoglycosides in combination with bacterial cell wall synthesis inhibitors, such as penicillin and vancomycin (Jordens *et al*., 1994; Klare *et al*., 1995; Devriese *et al*., 1996; van der Auwera *et al*.,1996; Zeana *et al*., 2001). Enterococci are now considered to be highly virulent due to their intrinsic ability to acquire antibiotic resistance determinants of several broad spectrum antibiotics and this allows them to cause super infections in patients already receiving antimicrobial therapy (Sahm *et al.*, 1997; Jones *et al.,* 1999). Moreover, enterococci, and especially the vancomycin-resistant enterococci (VRE), are now recognized as one of the most important cause of nosocomial infections in seriously ill and immuno-compromised patients (Patel, 2003).

Despite the fact that a number of species have been implicated *Enterococcus faecium* and *Enterococcus faecalis*, are the predominant species in which vancomycin resistance determinants are most often detected (Eaton and Gasson, 2001; Giraffa, 2002; Chingwaru, 2003; Klein, 2003; Gomes, 2008). Vancomycin-resistance results when vancomycin-sensitive *Enterococcus* strains obtain new DNA in the form of plasmids or transposons which encode genes that confer resistance to vancomycin (Courvain, 2006). Considering the fact that aminoglycosides are widely used for the clinical management of variety of infections, the emergence of bacterial resistance to these antibiotics compromises their utility (Todar, 2009). Moreover, bacterial resistance mitigates clinical efficiency in severe infections, thus creating a pressing need for the discovery and development of structurally novel and potent antibiotics against aminoglycoside-resistant strains (Walsh, 2003).

The increasing ability of bacteria to resist destruction by antibiotics and thus compromising the treatment of microbial infections has raised serious concern within the health care system (Cohen *et al*., 1992). The difficulty that is associated with the management of infections caused by vancomycin resistant enterococci has motivated extensive research in the area, and much is currently known about the diverse mechanisms employed by these organisms to avoid the deleterious effects of antimicrobial agents (Davies and Webb, 1998; Write *et al.,* 1999).

Antibiotic resistant enterococci and most especially VRE from animal species and food products such as beef and vegetables may enter the food chain and be transmitted to humans (Phillips *et al.,* 2004). Once these organisms are established in the gastrointestinal tract of their human hosts they may transfer their resistance determinants to other commensal bacteria species (Van den Bogaard *et al.,* 2002). Despite the fact that vancomycin is not commonly used in both human and veterinary medicine, recent studies have revealed a high prevalence of VRE in animal species, meat and water (Borgen *et al.,* 2001; Ateba and Maribeng, 2011). In the present study leafy vegetables from supermarkets are evaluated for the presence of vancomycin resistant enterococci.

2. Material and Methods

3. Materials and Methods

3.1 Area of study

3.1.1 Sample collection

Leafy vegetable samples were collected from some randomly selected retail shops in the Mafikeng area - Northwest Province, South Africa. Seventeen samples that comprised of 6 lettuce and 11 spinach were collected from small retail shops. The samples were properly labelled and transported on ice to the laboratory for analysis.

Laboratory analysis

3.2.1 Selective isolation of *Enterococcus* species

The lettuce and spinach samples were immediately analysed upon arrival in the laboratory. Approximately 1 gram portion of the leaves were placed into sterile sample collection bags, and washed with 5ml of 2% peptone water and 5% of vinegar, respectively. Aliquots of 100µl from the resulting solutions were spread-plated on Bile Esculin Agar (BEA), (Merck, South Africa). The plates were incubated aerobically at 37^{o}C for 24 hours and typical black colonies were considered as presumptive *Enterococcus* species. These isolates were sub-cultured on fresh BEA plates and the plates were incubated aerobically at 37^{o}C for 24 hours. The isolates were stored at room temperature and were subjected to biochemical tests for identification as enterococci.

3.3 Bacterial Identification

3.3.1 Gram stain

Gram staining that is used to distinguish between Gram-positive and Gram-negative bacteria was performed using standard methods (Cruikshank *et al.,* 1975). Isolates that were Gram-positive cocci were retained and later subjected to both preliminary and confirmatory identification tests for *Enterococcus* species.

3.4. Biochemical tests for confirming the identity of enterococci

3.4.1 Oxidase test

Oxidase test was performed using the oxidase test reagent from Pro-Lab Diagnostics – United Kingdom. When performing the test, a single colony was placed on a filter paper (Whatman International Ltd, Maidstone, England) and a drop of oxidase reagent was added to the culture. The two were mixed using a sterile wire loop and the results were read within 30 seconds. *Enterococcus* species are oxidase negative hence all isolates that satisfied this preliminary identification criterion were subjected to the catalase test.

3.4.2 Catalase test

The catalase test facilitates the detection of the enzyme catalase in bacteria. Catalase is a protective enzyme in bacterial species that is capable of destroying the chemical hydrogen peroxide which is dangerous. Catalase enzymes therefore decompose hydrogen peroxide to water and oxygen. Enterococci are usually catalase negative although weak positive results are commonly identified. In performing the test, a pure colony of an isolate was placed on a clean microscope slide and a drop of 2% hydrogen peroxide was added to the culture. Positive results were identified based on the formation of bubbles while lack of the formation of bubbles was recorded as negative results.

3.4.3 Extraction of DNA from potential enterococci

Genomic DNA was extracted from all presumptive enterococci using a modified cell boiling method. Fresh cultures were prepared by spread plating the isolates onto BEA plates to revive the cells. Plates were incubated aerobically at 37°C for 24 hours. After incubation, 500µl of sterile water was placed in 1.5 ml microfuge tube 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 digital dry bath (Biorad) 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.

3.4.4 PCR for the identification of enterococci species using 16S rRNA gene

Specific PCR for the amplification of enterococci specific 16S rRNA gene fragments were performed as previously described (Butterworth *et al.,* 2002). Primers used were E16SF and E16SR with sequences (5'-GGATTAGATACCCTGGTAGTCC-3') and (5'-TCGTTGCGGGACTTAACCCAAC-3') respectively (Butterworth; 2002). Reactions were performed in 25µl volumes that comprised 1X Master mix, 0.25 µl each primer, 5µl template DNA and nuclease free water. The amplifications were performed at 95°C for 4 minutes, 30 cycles of 95°C for 30 seconds, 58°C for 60 seconds, 72°C for 60 seconds and a final elongation step at 72°C for 7 minutes. The amplicons were held at 4°C until electrophoresis. Aliquots of, 5µl of the amplicons were separated by electrophoresis on a 1.5% (w/v) agarose gel using 1 X TAE. Each gel contained a 100bp molecular size standard ladder (Roche Diagnostics, Germany). The gels were run at 100V for 10 minutes and latter at 60V for 4 hours. The gels were stained in ethidium bromide (0.001µg/ml) for 15 minutes and amplicons were visualized under U.V light at 420nm wavelength (Sambrook *et al.,* 1989). A Gene Genius Bio Imaging System (Syngene, Synoptics; UK) was used to capture the image using Gene Snap (version 6.00.22) software. GeneTools (version 3.07.01) software (Syngene, Synoptics; UK) was used to analyse the images in order to determine the relative sizes of the amplicons.

3.4.5 Antibiotic resistance susceptibility test

The antibiotic resistant profiles of the isolates were determined using the Kirby-Bauer disc diffusion technique (Kirby *et al.,* 1966). *Enterococcus* isolates were screened using Mueller-Hinton agar (Merck, South Africa) as outline by the National Committee on Clinical Laboratory Standards (NCCLS, 2000). In performing the tests, bacterial suspensions were prepared and aliquots of 100µl from each dilution was spread-plated on Mueller-Hinton agar. The antibiotics that appear on Table 1 were placed on the inoculated agar and the plates were incubated aerobically at 37°C for 24 hours. The antibiotic inhibition zone diameters were measured and recorded. Standard reference values that occur in Table 1 were used to classify isolates as being susceptible, intermediate resistant and resistant to a particular antibiotic.

3. Results

3.1 Occurrence of enterococci in leafy vegetables (lettuce and spinach)

Seventeen samples were collected from shops in Mafikeng. These samples were analysed for the presence of *Enterococcus* species. The number of isolates obtained from the different samples is shown in Table 2. As shown in the Table, a total of 136 potential isolates were obtained based on colonial appearance and all these isolates were Gram positive cocci, negative for the oxidase tests and weakly positive for the catalase enzyme. Generally, all the samples were positive for enterococci and these isolates could have severe health implications in humans if the vegetables are consumed undercooked or raw as in the case of salads.

Table 1: Details of the antibiotics that is used in this study

Antibiotic	Abbrev	Discs conc. (µg)	Inhibitory zone diameter (mm)		
			R	I	S
Teicoplanin	TEC	30	≤10	11-13	≥14
Streptomycin	S	10	≤11	12-14	≥15
Ampicillin	AP	10	≤11	12-14	≥15
Erythromycin	E	15	≤13	14-22	≥23
Vancomycin	V	30	≤9	10-11	≥12
Ciprofloxacin	CIP	5	≤15	16-20	≥21
Amoxicillin	A	10	≤ 19		≥20
Tetracycline	TE	30	≤14	15-18	≥19
Doxycycline	DXT	30	≤12	13-15	≥16
Chloramphenicol	C	30	≤ 12	13-17	≥18
Norfloxacin	Nor	10	≤ 12	13-15	≥17

Table 2: Proportion of *Enterococcus* spp. isolated from the different sampling site

Samples	Gram stain (+ve) coccus	Oxidase test (-ve)	Catalase test
MP lettuce N=32	32	32	32
MF lettuce N=32	32	32	32
MS lettuce N=11	11	11	11
MT spinach N=61	61	61	61
Total No. of isolates	136	136	136

N=number of *Enterococcus* spp. isolated

4.2 Detection of eneterococci using PCR analysis

All the 136 isolates that were positive for the preliminary tests were subjected to confirmatory identification test for enterococci using the 16S rRNA specific PCR analysis. The number of isolates that were positively identified from the different samples are shown in Table 3. As shown in Table 3 a large proportion (60.3%) of enterococci were isolated from spinach when compared to lettuce (39.7%). A course for concern is the fact that lettuce is consumed raw in the form of salad in many homes and ready to

eat food outlets. It is therefore important to wash these food products properly before they are consumed.

Table 3: Proportion of *Enterococcus* spp. isolated from the different sampling site

Samples	Number of isolates tested	Number of isolates positive by 16S rRNA PCR assay
MP lettuce (NI=32)	32	14
MF lettuce (NI=32)	32	10
MS lettuce (NI=11)	11	7
MT spinach (NI=61)	61	47
Total No. of isolates	**136**	**78**

NI=Number of *Enterococcus* spp. isolated

4.3. The antibiotic susceptibility test of *Enterococcus* species isolated from lettuce

The isolates were tested to determine their antibiotic resistant profiles against eleven different antibiotics and the results are shown in Table 4. Generally a large proportion (72.1% to 100%) of the isolates were most often resistant to the antibiotics; amoxicillin, ampicillin, vancomycin, chloramphenicol, teicoplanin and erythromycin. On the contrary only small proportions (9% to 28.1%) of these isolates were resistant to tetracycline and doxycycline. These two antibiotics are tetracyclines that are used to treat a number of infections. Moreover, similarly small proportions (27.3% to 37.5%) of the isolates were resistant to ciprofloxacin. Ciprofloxacin is an antibiotic in a group of drugs called fluoroquinolones that is used to fight bacteria in the body. However, resistance shown against norfloxacin was higher than that observed against.

Table 4: The results of antibiotic susceptibility test of enterococci.

Samples		AP	TE	CIP	A	E	S	VA	TEC	DXT	C	Nor
MP lettuce	NR	32	8	12	32	28	17	31	29	9	29	11
NT=32	%	100	25	37.5	100	87.5	53.1	96.9	90.6	28.1	90.6	34.4
MF lettuce	NR	32	7	11	31	30	14	32	27	7	30	13
NT=32	%	100	21.9	34.4	96.9	93.8	43.8	100	84.4	21.9	93.8	40.6
MS lettuce	NR	11	1	3	9	6	2	9	8	2	10	5
NT=11	%	100	9.1	27.3	81.8	54.5	18.2	81.8	72.7	18.2	90.9	45.5
MT spinach	NR	61	60	18	61	61	50	61	61	57	44	11
NT=61	%	100	98.4	29.5	100	100	82	100	100	93.4	72.1	18

NR=Number resistant, NT=Number tested

4. Discussions

The relationship between the presence *Enterococcus* species and the levels of contamination in different foods in both developing and developed countries has been extensively reviewed (Franz *et al.,* 1999, and Giraffa, 2002). Moreover, the presence of enterococci in food products that harbour multiple antibiotic resistance and virulence gene determinants limit therapeutic options (Montecalvo *et al.,* 1994; Smith, 2002). Given the problems associated with the management of enterococci and particularly those that are resistant to multiple antibiotics it is therefore important to determine their presence in food and water sources.

The primary aim of this study was to isolate enterococci from leafy vegetables (lettuce and spinach). This was motivated from the fact that multiple antibiotic resistant enterococci have been isolated from groundwater intended for human consumption in the area. Leafy vegetables may be contaminated by various microorganisms through intentional or accidental inputs to the growing field environment and agents that contribute the contaminants include water, soil, animals and birds (Brackett, 1999; Beuchat, 2006). Moreover, the level of contamination could increase between the farm-to-consumer chain. Considering that the microbial quality of fresh produce such as vegetables is affected by chemical, physical and biological factors of the cultivar and the environment, it therefore means during the harvesting, packaging, transportation, handling and retail of vegetables hygiene practices should be implemented. In the present study, enterococci was isolated from all the samples using both preliminary and PCR methods.

Based on the fact that the microbial quality of fresh produce especially vegetables includes a combination of microbial activity, enzymatic activity, growth of the pathogen and metabolic byproducts that contribute to the visual and organoleptic quality (Sela and Fallik, 2009) it is important to implement strategies to reduce microbial contamination.

Moreover, the presence of enterococci in lettuce was a cause for concern since the leafy vegetable is usually consumed uncooked in the form of salad. Despite efforts made by food quality regulating bodies foodborne illness is still a major problem even in countries with more advanced health care systems and this is mainly due to the fact that microbes can enter the food chain at different stages and they are able to cope in environment and produce toxic substances (Havelaar *et al.,* 2010).

Another objective of the study was to determine the antibiotic resistance profiles of the enterococci isolates. A large proportion (72.7 to 100%) of isolates from lettuce in all the areas sampled were resistant to amoxicillin, ampicillin, vancomycin, chloramphenicol, teicoplanin and erythromycin. On the contrary, a small proportion (19.1 to 34.4%) of these isolates were resistant to tetracycline, norfloxacin, ciprofloxacin and doxycycline. A large proportion of *Enterococcus* species were resistant to three or more antibiotics and hence were termed multiple antibiotic resistant isolates (MAR). Similar observations had been reported in the area for erythromycin, chloramphenicol, amoxycillin and tetracycline (Moneoang and Bezuidenhout, 2009).

Tetracycline is easily accessible over the counter and hence the most commonly used antibiotic on animals in the area. However, the resistnce data for tetracycline does not really indicate the presence of a pre-exposed selective pressure. Vancomycin is not used in both veterinary and human medicine in the area. Thus the identification of Vancomycin resistant enterococci (VRE) was a cause for concern. VRE may pose a severe challenge to humans since it is very difficult to treat infections they cause. Constant monitoring of the antibiotic resistant profiles of enteroccoci in ground and recreational water sources could provide a comprehensive data of the resistant patterns of these pathogens in the area. This would improve information on treatment options for enterococcal infections in humans.

5. Conclusion

The present study evaluated the occurrence of multiple antibiotic resistant enterococci in leafy vegetables and the results indicated that all the samples were positive for the microbes. Moreover, aminoglycoside resistance was detected among a large proportion of enterococci. It is therefore suggested that these isolates may have severe health implications on consumers and this ignites the need to implement proper farm management and hygiene practices during the cultivation, harvesting, packaging and retail of these fresh produce.

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