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Full Length Research Paper

Characterisation of enterococci and *Escherichia coli* isolated from commercial and communal pigs from Mafikeng in the North-West Province, South Africa

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The aim of this study was to isolate enterococci and *Escherichia coli* from faeces collected from commercial and communal pigs, and to characterise these isolates using antibiotic susceptibility profiles. *Enterococcus* selective agar and eosin methylene blue lactose agar were used for enterococci and *E. coli* isolation, respectively. Gram staining, API 20 Strep and API 20E were used for identification of enterococci and *E. coli*, respectively. Three-hundred-and-four enterococci and 208 *E. coli* were identified. The most prevalent enterococci species were *Enterococcus faecium* (58%) and *Enterococcus gallinarum* (23%). A large proportion of enterococci (62.5% to 100%) and *E. coli* (88.5 to 100%) were resistant to erythromycin, oxytetracycline and sulphamethoxazole. No vancomycin-resistant enterococci were found and PCR analysis for *vanA*, *vanB* and *vanC-1* were all negative. Less than 7% of enterococci were resistant to ampicillin and amoxicillin, whereas 45% of *E. coli* isolates were resistant to the same antibiotics. Antibiotic susceptibility tests and clustering patterns showed some similarities among these isolates. From the results, a common origin of the isolates or histories of antibiotic use among these farms was proposed. It could also be concluded that vancomycin-resistant enterococci were not present in pigs on these two farms.

Key words: Vancomycin resistant enterococci, vancomycin susceptible enterococci, tetracycline, resistance genes, multiple antibiotic resistance.

INTRODUCTION

Enterococci form part of the normal flora of the intestinal tract of animals and humans (Blondeau and Vaughan, 2000; Klein, 2003). Among all the enterococci, *Entero-coccus faecium* and *E. faecalis* are the most prevalent species (Takeuchi et al., 2005), and significant cause of nosocomial infections (Blondeau and Vaughan, 2000). *Escherichia coli* also form part of the normal microflora of the gut of humans and animals (Naylor et al., 2005). Pathogenic *E. coli* strains have been recognised since the early 1900s, causing disease in both humans (Naylor et and animals (Blondeau and Vaughan, 2000).Multiple antibiotic resistance (MAR) is common among various microorganisms (Guan et al., 2002), and is nor-mally associated with the presence of antibiotic resistan-ce genes (Maynard et al., 2003; Bryan et al., 2004). Antibiotic susceptibility tests are used to generate MAR patterns (Rota et al., 1996) and MAR indices (Kaspar et al., 1990; Guan et al., 2002). These tests reflect the extent of selective pressures on the microbial floras of the gastro-intestinal tract of humans and animals imposed by excessive antibiotic use (Guan et al., 2002) . This technique could thus be used for classification and discrimination of isolates of the same or different location (Kaspar et al., 1990; Guan et al., 2002).

Enterococci have the ability to acquire and transfer resistance genetic markers to and from other bacterial species such as S*taphylococcus aureus* (Gambaratto et -

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al., 2000). In the recent past, vancomycin has been used as the last resort to treat enterococcal infections (Gambaratto et al., 2000). Vancomycin- resistant enterococci (VRE) are now endemic worldwide. It was firstly reported in Europe in 1986 and two years later in the United State of America (Perl, 1999). Furthermore, problems of VRE have also become a reality in South Africa (Derby et al., 1998; von Gottberg et al., 2000).

According to Blondeau and Vaughan (2000), resistance to vancomycin may occur due to mutations, transposons and resistance genes. Moreover, the extensive use of the growth promoter avoparcin in most of the European countries was also correlated to the high incidence of VRE (Wegener et al., 1999). In the United States, VREs were thought to occur primarily by the unskilful application of vancomycin in hospitals (Lemcke and Bulte, 2000).

In most cases, resistance to vancomycin is associated with the activity of the *vanA* gene, which confers high levels of vancomycin and teicoplanin resistance (Fluit et al., 2001). On the other hand, *vanB* confers moderate levels of vancomycin resistance and susceptibility to teicoplanin *in vitro* (Fluit et al., 2001). Another vancomycin resistance determinant is VanC. The genes encoding for this are intrinsic in *Enterococcus gallinarum* (*vanC- 1*), *E. casseliflavus* (*vanC- 2*) and *E. flavescens* (*vanC -3*) (Fluit et al., 2001). In the clinical environment, it may be imperative to determine the presence of these genes in order to establish the appropriate therapeutic and control measures (Hanaki et al., 2004). Studies have shown that PCR-based techniques are efficient and reliable for the surveillance of VREs (Bell et al., 1998; Fluit et al., 2001). The goal of this study was to isolate and characterise enterococci and *E. coli* from a commercial and a communal pig farm in the North-West Province (RSA). The purpose was to determine the antibiotic resistance patterns of these isolates, and to screen for the presence of VRE or genes responsible for vancomycin resistance among the enterococci isolates.

MATERIALS AND METHODS

Sampling and methodology

Two hundred faecal samples were collected from Mareetsane commercial pigs and the same number collected from Tlapeng communal pigs. All the samples were collected using sterile gloves directly from the rectum. Faeces (1 g) was inoculated into nutrient broth (5 ml) and incubated at 37°C for 24 h. These samples were sub-cultured on *Enterococcus* selective agar and eosin methylene blue lactose agar (Merck, RSA) for enterococci and *E. coli* isolation, respectively. The plates were incubated at 37°C for 16 - 24 h. Colonies showing typical characteristics of entero-cocci (black) and *E. coli* (metallic sheen) were selected for further analysis. Gram Staining was performed, then API 20 Strep and API 20E (bioMêrieux, France) to identify enterococi and *E. coli*, respect-tively.

Characterisation of isolates by MAR phenotypes and MAR indices

The antibiotic susceptibility test was conducted by the disc - diffusion method in Mueller-Hinton agar with and without 5% sheep blood for enterococci and *E. coli* , respectively (Kirby-Bauer, 1966) . The anti-biotics used are listed in Table 2 and abbreviations of antibiotics were according to the manufacturer (Mast Diagnostics, U.K.). Multi-ple antibiotic resistance phenotypes were determined for all the iso-lates that were resistant to three and more antibiotics (Rota et al., 1996). The MAR indices of isolates of a particular sampling station were also generated (Kaspar et al., 1990).

DNA extraction and PCR

Genomic DNA was extracted from selected MAR enterococci, *E. coli* and vancomycin-susceptible enterococci using a modified CTAB-PVP DNA extraction method (Doyle and Doyle, 1990). Briefly, this included incubation in CTAB-PVP extraction buffer, followed by chloroform:isoamyl alcohol (24:1) extraction and precipitation of DNA using an ethanol and sodium chloride step. The extracted DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]).

The 16S rRNA primer pair (Table 1) was used to amplify 16S rRNA gene fragment (Positive control). The same DNA was then used for screening of vancomycin resistance gene fragments using primer pairs as indicated in Table 1. Primers were synthesised by Inqaba Biotech (Pretoria, RSA). All the amplifications were performed in a PTC-200 DNA Engine ^{TM} System (Bio-Rad, UK) . The PCR mix (25 µl) for amplification of 16S rRNA consisted of 100 ng of DNA containing 2X PCR master mix, 25 mM $MgCl_2$, 16S primer (50 pmole), RNAse- DNAse free water (Fermentas, US) with the add-ition of 1 U Taq polymerase (Promega, USA). The PCR conditions consisted of an initial denaturing period of 5 min at 95°C, followed by 35 cycles of the following: 95°C for 30 s (denaturing), 55°C for 30 s (annealing) and 72°C for 60 s (elongation), with a final elonga-tion step of 72°C for 5 min.

For vancomycin resistance gene amplification, each PCR cycle consisted of an initial denaturing period of 2 min at 94°C, followed by 35 cycles of the following: 94°C for 60 s (denaturing), 62°C for 60 s (annealing) and 72°C for 60 s (elongation), with a final elongation step of 72°C for 10 min (Bell et al., 1998). Another set of PCR reaction conditions were also tested and consisted of 35 cycles at 94°C for 60 s (denaturing), 50°C for 60 s (annealing), 72°C for 60 s (elongation) and a final elongation step at 72°C for 10 min (Donabedian et al., 2000).

PCR products were resolved by gel electrophoresis on 1% (w/v) agarose that contained ethidium bromide (0.001 µg/µl). The electrophoresis buffer was 1X TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Electrophoresis was performed at 60 V for 60 min. A Gene Genius Bio- Imaging System (Syngene, Synoptics, UK) was used to capture the image using GeneSnap (version 6.00.22) software.

Statistical analysis

The complete antibiotic inhibition zone data set of both enterococci and *E. coli* were separately analysed using Minitab Release (version 13.31, France). These data sets were subjected to Pearson's correlation to determine the relationship of all the enterococci and *E. coli* isolated from the two farms. The correlation was considered significant at $P<0.01$. Furthermore, a pooled data set of the total antibiotic inhibition zone diameters for *E. faecium* and *E. coli* were exported to Statistica (version 7; StatSoft software, US). The data were analysed by multivariate exploratory techniques using the Ward's clustering method and Euclidean distances. The resultant dendrogram indicated clustering patterns and relationships (Berge et al., 2003).

Table 1. Oligonucleotide primers and their sequences used in this study.

Primer	Gene fragment	Sequence	Reference
VanAF		5 GTA GGC TGC GAT ATT CAA AGC 3	
VanAR	vanA	5 CGA TTC AAT TGC GTA GTC CAA 3	Bell et al., 1998
VanBF		5 GTA GGC TGC GAT ATT CAA AGC 3	
VanBR	vanB	5 GCC GAC AAT CAA ATC ATC CTC 3	
VanC1F		5 TGG TAT TGG TAT CAA GGA AAC C 3	
VanC1R	vanC-1	5 AGA TTG GAG CGC TGT TTT GTC 3	
GM5F		5 TAC GGG AGG CAG CAG 3	
907 R	16S	5 CCG TCA ATT CCT TTG AGT TT 3	Muyzer et al., 1995

Table 2. The numbers and percentages of various enterococci species and *E. coli* isolated from Mareetsane commercial pigs and Tlapeng communal pigs that were resistant to different antibiotics.

		Mareetsane commercial pigs									
Microorganism		**AP 10µg**	**A 10µg**	**E 15µg**	**OT 30µg**	**SMX 200U**	**KF 30µg**	**NE 30µg**	**GM 30µg**	**VA 30µg**	**C 30µg**
E. faecium	NR	3	1	64	86	86	74	58	75	0	-
NT = 86	%	3.5	1.2	74.4	100	100	86.0	67.4	87.2	0	-
E. gallinarum	NR	2	1	24	30	30	21	19	27	0	-
NT = 31	%	6.5	3.2	77.4	96.8	96.8	67.7	61.3	87.1	0	-
E. durans	NR	0	0	2	8	8	0	2	5	0	-
NT = 8	%	0	0	25	100	100	0	25	62.5	0	-
E. avium	NR	0	0	4	6	6	1	2	4	0	-
NT = 6	%	0	0	66.7	100	100	16.7	33.3	66.7	0	-
E. coli	NR	29	31	95	99	92	23	33	8	-	18
NT = 104	%	27.9	29.8	91.3	95.2	88.5	22.1	31.7	7.7	-	17.3
		Tlapeng communal pigs									
E. faecium	NR	1	0	45	57	64	43	46	44	0	-
NT = 64	%	1.6	0	70.3	89.1	100	67.2	71.9	68.8	0	-
E. coli	NR	8	8	46	46	46	4	0	0	-	2
NT = 46	%	17.4	17.4	100	100	100	8.7	0	0	-	3

NT (number tested), NR (number resistant), A (amoxicillin), AP (ampicillin), GM (gentamycin), OT (oxytetracycline), NE (neomycin), SMX (sulphamethoxazole), KF (cephalothin), E (erythromycin), VA (vancomycin), C (chloramphenicol).

RESULTS

Bacterial species identification

Three-hundred-and- four (76%) enterococci and 208 (52%) *E. coli* were positively identified from 400 faecal samples. During this study, *E. faecium* was the dominant species isolated and constituted 48.5% (Tlapeng) and 58% (Mareetsane) of the total enterococci population. The level of *E. gallinarum* was 9% among the Tlapeng isolates and 23% among the Mareetsane isolates. The other enterococci species contributed between 0.5 and 4% to the total enterococci population from both pig farms and the rest could not be identified to species level.

Antibiotic susceptibility tests

The activities of nine different antibiotics were tested on both enterococci and *E coli*, and the results are represented in Table 2. A large proportion of enterococci isolates (62.5 - 100%) showed resistance to oxytetracycline, sulphamethoxazole, gentamycin and erythromycin. Among the enterococci, 70.3 to 100% of *E. faecium* isolated from both farms were resistant to erythromycin, oxytetracycline and sulphamethoxazole. A considerable percentage of

these *E. faecium* isolates were resistant to aminoglycosides (67.4 - 87.2%) and cephalothin (67.2 - 86.0%). Less than 7% of enterococci isolates were resistant to -lactam antibiotics (ampicillin and amoxicillin). All the tested enterococci from Mareetsane and Tlapeng pigs were susceptible to vancomycin.

To establish whether the genes responsible for vancomycin resistance (particularly low levels) might be present in the enterococci populations from Mareetsane and Tlapeng pig farms, PCR assays were employed. The genes targeted included *vanA*, *vanB* and *vanC-1* . All the identified *E. faecium* were screened for presence of *vanA* and *vanB* genes, and 50% (8 from 16) of *E. gallinarum* from both pig farms were screened for presence of *vanC-1* gene. None of the enterococci species tested harboured either the *vanA*, *vanB* and/or *vanC -1* genes (results not shown). However, only an image of the positive control (16S rRNA amplicons) was depicted in Figure 3.

Susceptibility profiles for *E. coli* showed that the majority (88.5 - 100%) was resistant to erythromycin, oxytetracycline and sulphamethoxazole (Table 2). Similar results were observed in enterococci isolates (Table 2). Levels of -lactam (ampicillin, amoxycillin and cephalothin)-resis-tant *E. coli* isolated from faeces of pigs in Mareetsane and Tlapeng ranged from 17.4 to 29.8% (Table 2).

MAR phenotypes and MAR indices

During this study, 150 *E. faecium* (86 and 64) and 122 *E. coli* (76 and 46) isolated from Mareetsane commercial and Tlapeng communal pig farms, respectively, were characterised for the MAR phenotypes and MAR indices. Ten dominant MAR phenotypes were observed from the 150 *E. faecium* tested. The predominant MAR phenotype was E-OT-SMX-KF-NE which occurred in 36% of the Mareetsane isolates, and E- OT-SMX-KF in 43.8% of the Tlapeng isolates. Thus, the majority of *E. faecium* isolates from both farms were resistant to E-OT-SMX -KF. The MAR indices were almost similar for Mareetsane and Tlapeng *E. faecium* isolates (0.5503 and 0.5121, respectively). Six dominant MAR phenotypes were found among 122 *E. coli* isolated from Mareetsane and Tlapeng pigs. The predominant MAR phenotype was E-OT-SMX, occurring in both Mareetsane (36.8%) and Tlapeng (65.2%) isolates. The MAR indices for Mareetsane and Tlapeng *E. coli* isolates were 0.4369 and 0.3864, respectively.

Clustering analysis

A total of 92 *E. faecium* isolates, as well as 92 *E. coli* were selected from both Mareetsane and Tlapeng pigs (46 per sampling station) in order to generate a dendrogram. Among all 46 isolates selected, 23 were from the dominant MAR phenotypes. The other 23 isolates were from the least dominant MAR phenotypes. This selection criterion was decided upon to maximise the potential for determining similarities and differences between the two pig farms. The results of the clustering analysis for both *E. faecium* (92) and *E. coli* (92) are presented in Figures 1 - 3, respectively. Figure 1 indicates 5 major clusters from the 92 *E. faecium* isolated from Mareetsane and Tlapeng pigs. Cluster I and III were mostly represented by Mareetsane isolates (78.3 and 57.1%, respectively). Clusters IV and V were represented mostly by Tlapeng isolates (78.9 and 76.9%). However, cluster II was formed by equal proportions (50%) of *E. faecium* from both farms, suggesting a close relationship of these isolates. Four major clusters were observed from *E. coli* isolated from both farms (Figure 2). Briefly, cluster I was mainly made up of Mareetsane isolates (93.8%), cluster II and cluster IV of Tlapeng isolates (73.1 and 63.2%, respectively). In cluster III a mixture of Mareetsane and Tlapeng isolates (54.8 and 45.2%), respectively, were found.

DISCUSSION

During this study enterococci and *E. coli* were isolated from faeces collected from both commercial and communal pigs. However, the numbers of these isolates were lower than the number of samples collected, since APIs could not confirm the identities of some of the other putative isolates. Results such as these are not uncommon and have also been reported previously (Hayes et al., 2003).

E. faecium was the most dominant enterococcal species identified in this study. The observation of this study is in agreement with Klein (2003) in which *E. faecium* was the most frequent species isolated from pigs. High levels of *E. faecium* were also observed among the 981 retailed raw meat (chicken, turkey, pork and beef) samples randomly obtained from 263 grocery stores in Iowa. Among all these meat samples, 61% of the isolates were *E. faecium*, 29% *E. faecalis* and 0.7% *E. gallinarum* (Hayes et al., 2003).

A large proportion of enterococci were resistant to multiple antibiotics, with resistance mostly shown to erythromycin, oxytetracycline and sulphamethoxazole. These results suggest a possibility of high use or exposure of these antibiotics within these pig farms. Less than 7% of all the enterococci isolates were resistant to -lactams (ampicillin and amoxicillin), indicating that these products could still be useful for enterococcal infection, particularly on the farms sampled.

During this study, large proportions of enterococci were resistant to the macrolide antibiotic (erythromcin). However, all of these isolates were susceptible to glycopeptide (vancomycin). In most of the European countries, the prevalence of erythromycin- and vancomycin-resistant bacterial species is mostly associated with the use of growth promoters such as the macrolide, tylosin (Boerlin et al., 2001) and the glycopeptide, avoparcin (Wegener et al., 1999). Additionally, studies have shown that cross-

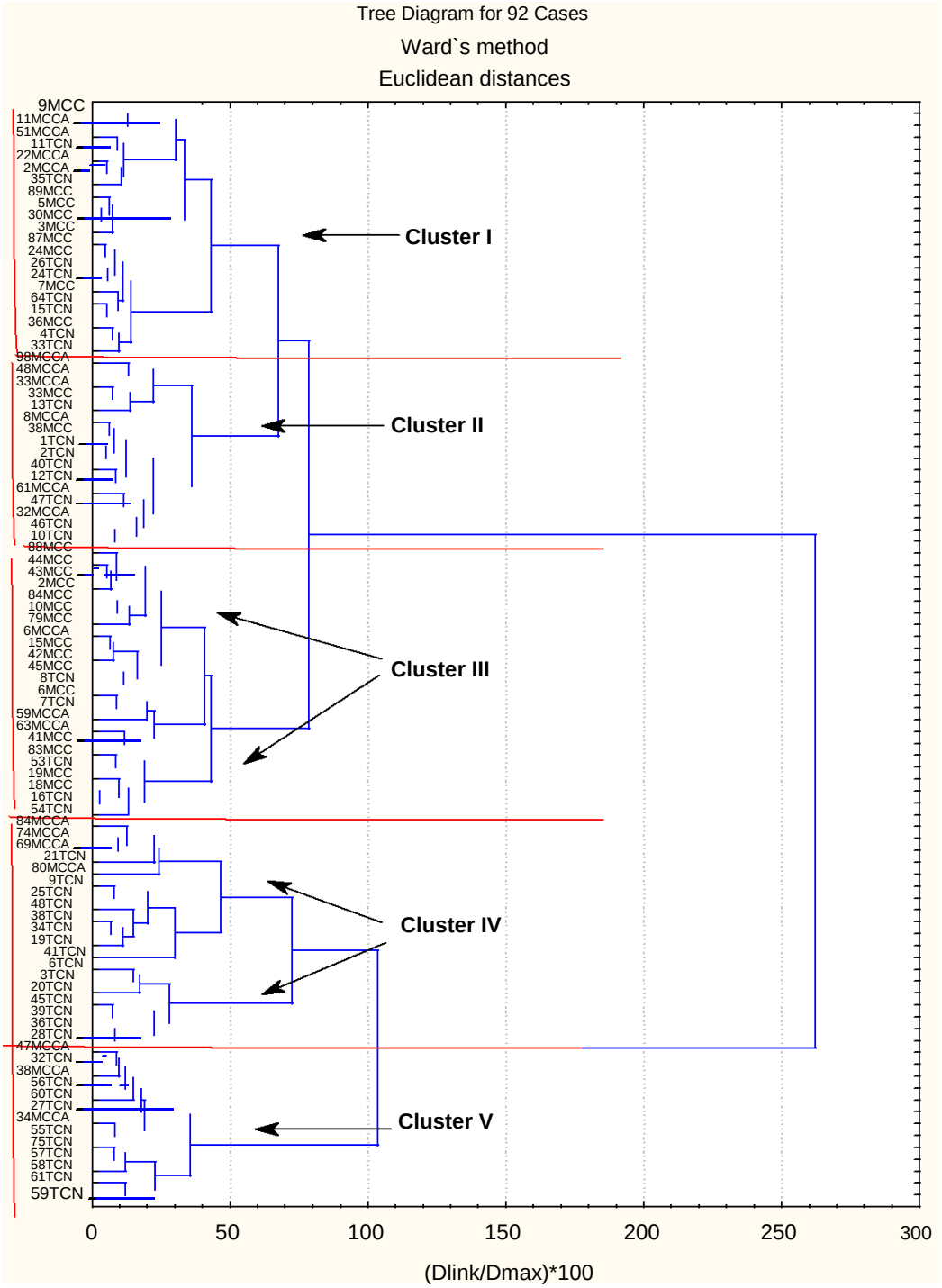

Figure 1. A dedrogram showing clustering patterns for 92 *E. faecium* isolated from Mareetsane and Tlapeng pigs. The analysis is based on complete antibiotic inhibition zone diameter data. Designation: MCCA; MCC (Mareetsane isolates), TCN (Tlapeng isolates).

selection and genetic linkage between erythromycin and vancomycin resistance occurs particularly when tylosin is used as a growth promoter (Boerlin et al., 2001; Jackson et al., 2004). *E. faecium* from these pigs were resistant to both erythromycin and vancomycin (Boerlin et al., 2001). In our study, a large proportion of enterococci isolates from both farms were resistant to erythromycin. This is a cause for concern. Even so, all the tested enterococci

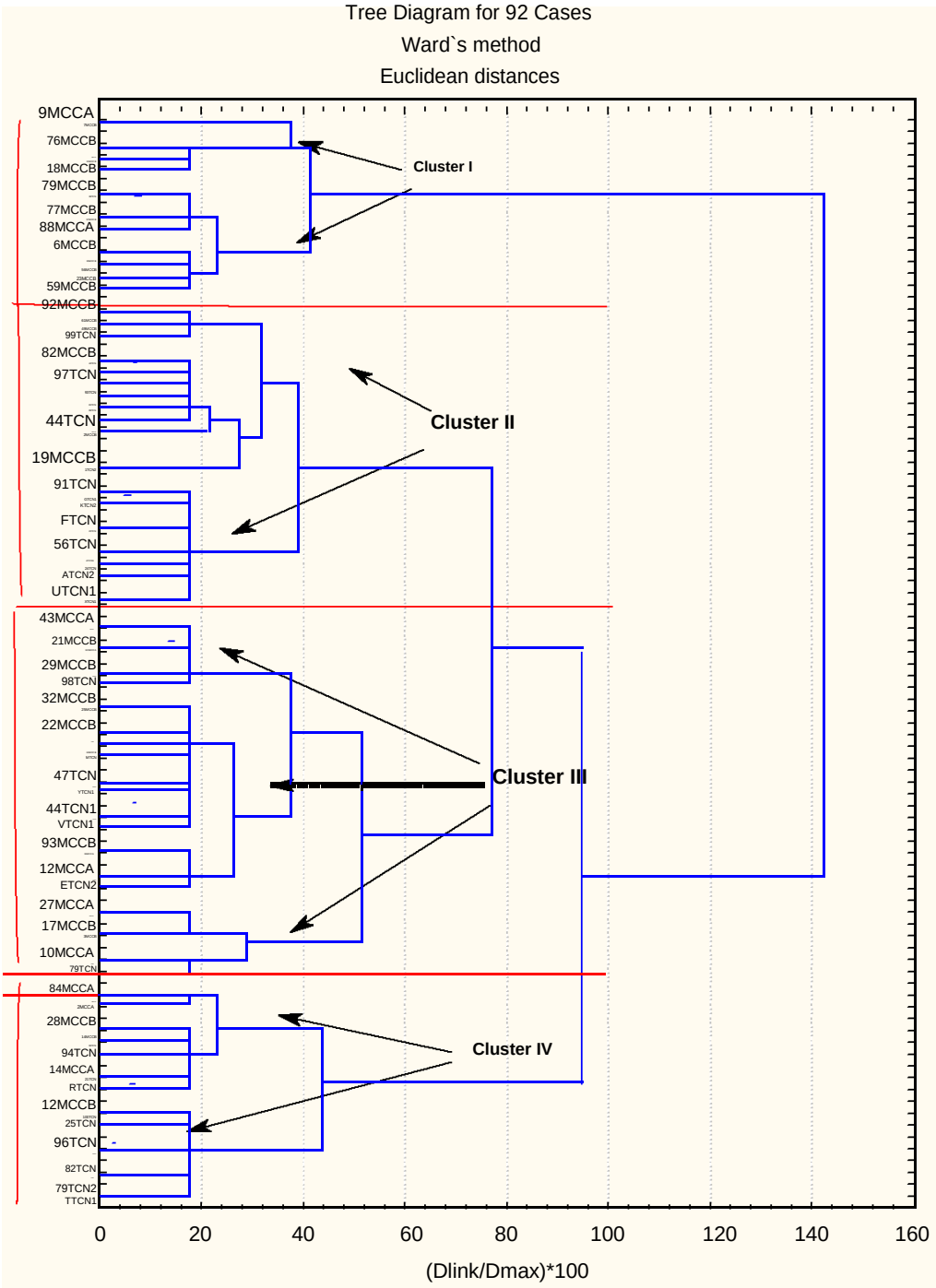

Figure 2. A dendrogram showing clustering patterns for 92 *E. coli* isolated from Mareetsane and Tlapeng pigs. The analysis is based on complete antibiotic inhibition zone diameter data. Designation: MCCA; MCCB (Mareetsane isolates), TCN, TCN1, TCN2 (Tlapeng isolates).

from Mareetsane and Tlapeng pigs were susceptible to vancomycin. The absence of VRE is not uncommon. Vancomycin - susceptible enterococci (VSE) were observed from faecal samples collected from pigs in Sweden (van den Bogaard et al., 2000). Such a trend was also observed among enterococci isolated from retailed raw meat products in Iowa (Hayes et al., 2003).

The farmers from both Mareetsane commercial and Tlapeng communal settings indicated that they do not use any growth promoters (tylosin and/or avoparcin) on

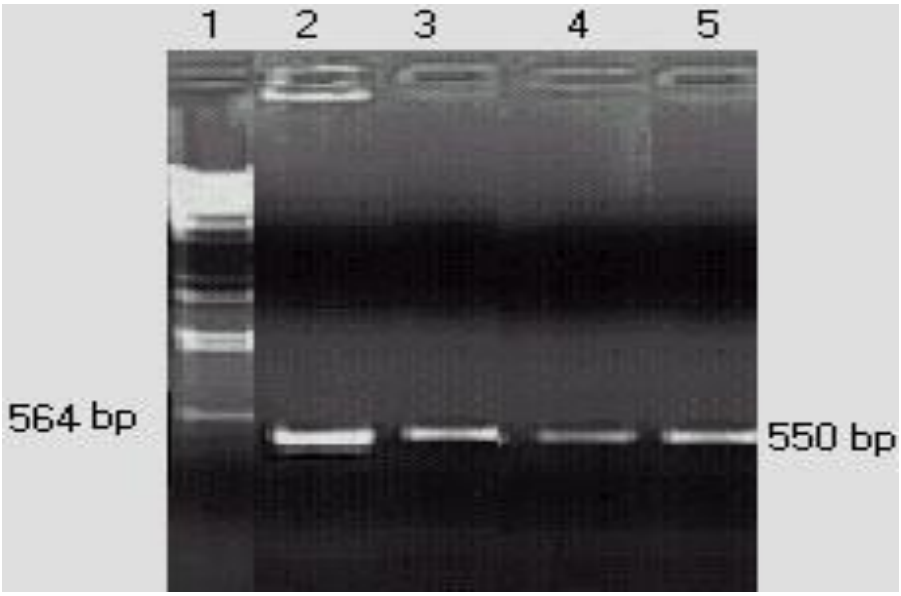

Figure 3. Image of a composite agarose gel showing an amplified 16S rRNA gene fragment from enterococci and *E. coli*. Lane 1 (Lambda DNA-Hind III digest), Lanes 2-3 (16S rRNA; enterococci), Lanes 4-5 (16S rRNA; *E. coli*).

their farms. They also indicated that they do not use vancomycin or any glycopeptides antibiotics for therapeutic purposes. Thus, the results from this study support their claims. When antibiotic resistance data sets of *E. faecium* isolated from the two farms were compared, a strong positive correlation ($r = 0.992$; $p < 0.001$) was observed between these data sets. This result suggested that pigs from these two farms had a common history of antibiotic exposure.

Commercial and communal farmers in the North-West Province, particularly those from areas around Mafikeng, have indicated that they rely on tetracycline for therapeutic treatment of their animals. Tetracycline is attractive to these farmers not only because of being a broad spectrum antibiotic, but also because it is a freely available over-the-counter product which is cost-effective. The results of the present study underscore the hypothesis that the continuous use of an antibiotic results in bacterial tolerance to that product. As such, high numbers of *E. coli* resistant to this antibiotic were isolated. This poses a potential threat to both animals and human health sectors.

In a study conducted in Canada, *E. coli* isolated from pigs were resistant to oxytetracycline (Maynard et al., 2003; Bryan et al., 2004). Previous studies showed that a considerable large percentage of *E. coli* isolated from animals such as chickens, cattle, goats and sheep (Bryan et al., 2004), as well as in human isolates (Bartoloni et al., 2006) were resistant to tetracycline.

A considerable percentage of *E. coli* isolated from pigs from both farms showed resistance to ß-lactam antibiotics (Table 2). Maynard et al. *(*2003) observed that between 15 - 30% of ETEC strains isolated from pigs in Canada were also resistant to these ß-lactams. Resistance to such antibiotics among *E. coli* isolated from pigs from Mareetsane and Tlapeng could be due to mutations in the penicillin-binding proteins and/or presence of bla_{TEM} resistance genes (Fluit et al., 2000). The higher percen-tages (29.8%) of these *E. coli* were resistant to the newer generation ß-lactam antibiotic, cephalothin, and this is a cause for concern. When comparing resistance results of *E. coli* isolated from Mareetsane and Tlapeng pig farms, a strong positive correlation ($r = 0.992$; $p < 0.001$) exis-ted, once again indicating common antibiotic usage regi-mes on these farms.

During this study, a large percentage of enterococci and *E. coli* showed multiple antibiotic resistances. The most dominant MAR phenotype pattern was E-OT-SMX-KF-NE for Mareetsane *E. faecium* isolates with only the absence of one antibiotic (NE) for Tlapeng isolates. The prevalent MAR phenotype for *E. coli* isolated from both pig farms was E-OT- SMX. During this study, it appeared that E-OT-SMX was the basis of all the phenotypes in both *E. faecium* and *E. coli*, suggesting a possibility of high use or exposure of these antibiotics in these pig farms. According to Kaspar et al. (1990), isolates with similar MAR phenotypes may have common origin or similar antibiotic exposure histories. Furthermore, the MAR phenotypes may sometimes suggest the extent of antimicrobials use in specific food animal production environments (Hayes et al., 2003).

When comparing the relationship of both *E. faecium*

and *E. coli* using the complete antibiotic inhibition zone diameter data by the clustering patterns (Figures 1 and 2), some close relationship between these isolates from Mareetsane and Tlapeng existed. A similar situation was shown by these isolates when considering the results of the resistance patterns (Table 2), MAR phenotypes and MAR indices. These results support the deduction that the isolates from both farms may have a common origin. Alternatively, these isolates may have had similar histories of antibiotic exposures.

Conclusion

Enterococci and *E. coli* were isolated and characterised in this study. It could be concluded that VREs are currently not present in pigs on the farms that were tested. This observation is encouraging, particularly for the marketing of pork from these farms. However, further studies are essential to screen more farms in the North-West Province to confirm the absence of VRE among pigs in this region. Furthermore, a large proportion of enterococci were susceptible to amoxycillin and ampicillin. These results indicate that -lactams could still be used as a synergy with other cell wall- active antibiotics such as vancomycin in the treatment of enterococcal infections in pigs, particularly in the areas sampled in the North-West Province (RSA).

The observations of this study provide some baseline data for management of antibiotic resistance among pigs in North-West Province (RSA). A large proportion of enterococci and *E. coli* showed MAR, and this should be considered a cause for concern for veterinary health authorities. This also suggests the need for changes in the use of antibiotics in both veterinary and human medicines, as some unusual events may affect propagation of antibiotic-resistant bacteria in the food environment and food supply.

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