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Efflux Activity in Multi-drug Resistant Enterococci from Environmental Samples

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Abstract

This study evaluated the genetic determinants of resistance identified in multi-drug resistant enterococci isolated from poultry and aquaculture samples. Thirty-nine enterococcal isolates identified as multi-drug resistant in previous studies were used. Polymerase chain reaction (PCR) and gel-electrophoresis methods were used to screen for vancomycin resistance genes (vanA and vanB) and plasmids. Further screening on antimicrobial resistance mechanism was carried out by adopting ethidium bromide-agar cartwheel method for efflux activity. This was confirmed by treating isolates with efflux pump inhibitor, carbonyl cyanide m-chlorophenylhydrazone (CCCP). The PCR test revealed the absence of plasmids and resistance genes. There was however over expression of efflux system in some isolates. The cell efflux capacity increased with increasing ethidium bromide concentration, which produced fluorescence of the bacterial mass. After confirmation with CCCP, only 61.54 % of the isolates expressed resistance for vancomycin, 58.97 %, 48.72 %, 48.15 %, and 28.21 % for ciprofloxacine, oxacillin, tetracycline, and gentamicin respectively. The highest increase in zones of inhibition after treatment with CCCP was seen in gentamicin where 71.79 % of the isolates had become susceptible as against 23.08% previously. Fourteen (35.89 %) isolates expressed active efflux for more than two antimicrobials each, while three and six of these expressed active efflux were for all five and four of the antimicrobials tested respectively. These results infer a high prevalence of multidrug resistance among enterococci isolates from the environmental samples, which was probably due to the presence of an active efflux system. These environments may serve as reservoir and vehicle to transport these resistant bacteria and genes to humans and other bacteria of public health significance.

Keywords: Antimicrobial resistance, efflux activity, efflux inhibitor, enterococci, reservoir

1. Introduction

Antimicrobial resistance is the ability of microorganisms such as bacteria, viruses, fungi and parasites to withstand the effects of antimicrobials and render the medications used to cure the infections they cause ineffective (Lukasova and Sustackova, 2003). This phenomenon has become a world-wide problem in human and veterinary medicine and is a major threat to public health. It is now generally accepted that the main risk factor for the increase in antibiotic resistance is an extensive use of antibiotics, which has led to the emergence and dissemination of resistant bacteria and resistance genes in animals and humans, where antibiotics are used for therapy and prophylaxis of infectious diseases (Lukasova and Sustackova, 2003; Anthony *et al*., 2000).

Most of the antibiotics used for agricultural purposes are only partially metabolized by animals and are then discharged through fecal contents either into sewage disposals or directly into rivers near animal farms. Consequently, the antibiotics used in agriculture are responsible for the increase in the prevalence of multiple antibiotic resistance (MAR) in animal farming and aquatic environments and may be directly linked to the antibiotic resistance problems in humans either via direct contact or through the food chain (Rho *et al*., 2011). The nutritive and therapeutic antibiotic treatment of farm animals amounts to a half of the world's antibiotic output and has also resulted in antibiotic-resistant bacteria (Dzidic *et al.,* 2008). Evidence is accumulating to support the hypothesis that antibiotic-resistant bacteria from poultry, pigs and cattle enter the food supply, can be found in human food, colonize human digestive tract and transfer resistance genes to human commensals (Dzidic *et al.,* 2008; Silbergeld *et al*., 2008).

Antimicrobial resistance in bacteria may emerge by one of several pathways: a bacterium of a normally susceptible species might become resistant by mutation or acquisition of new genes. Some bacterial species are normally and inherently resistant to certain antibiotics whereas others are susceptible. Susceptibility has three requirements: a target for reaction, a mechanism for transport into the cell before the antibiotic action takes place and absence of enzymes that could inactivate or modify the antibiotic. A change in any of these prerequisites could render an antibiotic-susceptible bacterium resistant to the drug (Lukasova and Sustackova, 2003).

Active efflux, an important component of bacterial resistance to most classes of antibiotics is mediated by efflux pumps, which are membrane-associated active transporters promoting the extrusion of toxic compounds, including antibiotics, from the cells (Zechini and Versace, 2009). Efflux pumps reduce the accumulation of antibiotics inside of the bacterial cells, and the slow phase in which the process of antibiotic efflux takes place provides sufficient time for the bacterium to adapt to the antibiotics and become resistant through mutations or alteration of antibiotic targets (Kumar and Varela, 2012). Efflux pumps are known to affect all classes of antibiotics, especially the macrolides, tetracyclines and fluoroquinolones because these antibiotics inhibit different aspects of protein and DNA biosynthesis and therefore must be intracellular to exert their effect (Dzidic *et al.,* 2008).

Enterococci are among the most antibiotic resistant bacterial pathogens known. For reasons not well understood, they appear to have served as a key collection point for a wide variety of antibiotic resistance determinants, and they disseminate these to other species. According to Miller *et al.,* (2014), multidrug resistant enterococci have developed resistance to almost all antimicrobials currently in use in clinical practice, by applying a wide array of genetic strategies including over expression of efflux pumps that promotes their survival.

This paper reports the possible involvement of active efflux systems in reduced susceptibility to gentamicin, oxacillin, tetracycline, ciprofloxacin and vancomycin antibiotics among resistant enterococcal isolates from chicken and aquaculture samples.

2. Materials and Method

2.1. Isolation and Identification

Thirty-nine antimicrobial resistant enterococci isolates from poultry and fish ponds in previous studies were used (Ikeme *et al.,* 2015, Chikwendu *et al.,* 2017). The isolates were cultured on Slanetz and Bartley agar (Oxoid, CM UK) at 44^0C for 48 h and identified based on the morphological characteristics. Enterococci appeared as typical red or pink colonies on Slanetz and Bartley agar. Isolates were preliminarily grouped based on their morphology, ability to grow

in the presence of triphenyltetrazolium chloride (TTC) and in the presence of 40 % bile salt, motility, catalase production, hemolysin production, pigmentation, growth at 10°C, 45°C and growth in 6.5% NaCl. The second step was identification of enterococci species following the recommendations of Manero and Blanch, (1999) by using their fermentation properties on sucrose, mannitol, arabinose, lactose, glucose, maltose, and sorbitol.

2.2. Antibiotic susceptibility testing

Antibiotic susceptibility testing was conducted on isolates using 10 antibiotics belonging to seven antibiotic classes. All isolates were tested for susceptibility to different antimicrobials using the Kirby-Bauer disk diffusion method as described by the National Committee for Clinical Laboratory Standard Guidelines (CLSI, 2007) and Bauer *et al*., (1966). Each of the Enterococci isolate was inoculated into nutrient broth overnight at 37°C before testing. The turbidity of the actively growing culture was adjusted to correspond with that of a barium sulphate (0.5 McFarland standards) standard. Subsequently 0.1 ml of the nutrient broth culture was inoculated onto Mueller Hinton agar plates (90mm diameter disposable petri dishes) and spread over the surface with sterile cotton swabs. The antibiogram consisted of 10 antibiotics (Oxoid, CM UK) encompassing seven antibiotic classes: penicillin (oxacillin-OXA, penicillin G-PEN), glycopeptides (vancomycin-VAN), fluoroquinolone (ciprofloxacin-CIP), macrolide (erythromycin-ERY), streptogramin (quinipristine-dalfopristine-Q/D), aminoglycoside (streptomycin-STR, kanamycin-KAN, gentamicin-GEN) and tetracycline (tetracycline-TET) were then placed on the surface of each plate by means of antibiotic disk dispenser. Five discs were placed in each petri dish. Within 15 minutes of the application of the discs, the plates were inverted and incubated at 37°C. After 18 h of incubation, inhibition zone diameters were measured using a transparent ruler and correlated into sensitive (S), or resistant (R) based on the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2007).

2.3. Molecular Characterization

2.3.1. Plasmid Isolation

Two methods were employed for each isolate. Plasmid extraction was carried out using the method described by Ehrenfeld and Clewell (1987) and Zhou *et al*., (1990) with slight modification. Pure isolates were inoculated on nutrient agar and incubated. The grown cells were suspended in 300 µl of STET solution (100 mM sucrose-50 mM, tris hydrochloride (pH 8)-10 mM, EDTA) and homogenized using thermo mixer buffer of the shaker for 15 min at 37^0C. 15 µl of lysozyme (10 µg/ml) was added and mixed immediately by vortexing for 5 sec. The eppendorf tubes were incubated for 5 min at 37^oC in an incubator. The tubes were placed in a boiling water bath for 1 min at 99^0C and then centrifuged at maximum speed (14,000 rpm) in a micro-centrifuge for 15 min at 4°C. The supernatant containing plasmid was transferred promptly in new eppendorf tubes, and then 200 µl of isopropanol was added in the supernatant and mixed by inverting the tube 4 - 6 times. It was incubated in the freezer for 30 min and centrifuged at maximum speed (14,000 rpm) for 15 min. The supernatant was discarded completely and 500 µl of 70% ethanol was added to the pellets. The tubes were closed, inverted several times and centrifuged at 14,000 rpm (maximum speed) for 10 min. The supernatant was discarded, blotted and dried. The plasmid DNA was then dissolved in 50 µl TE- (Tris hydrochloride 10 mM, EDTA 1 mM) (pH 8.0) and stored at 4^0C.

The second method described by Zhou *et al*., (1990) was adopted which involves the use of TENS solution (Tris 25 mM, EDTA 10 mM, NaOH 0.1 N and SDS (sodium dodecyl sulphate) -

0.5%) instead of STET solution. The plasmid DNA were electrophoresed through a 0.8%% agarose gel stained with ethidium bromide for 40 min at 80V and photographed under UV light.

2.3.2. Enterococcal DNA Extraction

DNA extraction was carried out using the method described by Li *et al*., (2003). Pure isolates were inoculated on nutrient agar and incubated overnight at 37^0C. Eppendorf tubes (1.5 ml) were labeled appropriately and 1 ml of sterile water was dispensed into them. The grown cells were suspended in 1 ml of DNase-RNase-free distilled water and vortexed. The cell suspension was centrifuged for 5 min at 14,000 rpm. The supernatants were discarded carefully and blotted on the paper towel provided. The pellets were resuspended in 200 µl DNase-RNase-free distilled water by vortexing to homogenize the pellets. The cell suspension was heated at 100^0C for 10 min and then centrifuged at 14,000 rpm for 5 min. The supernatants were carefully transferred to a new microcentrifuge tube by gentle aspiration using a micropipette and then chilled immediately on ice. An aliquot of 1.5 ml of the supernatant was used as the template DNA in the polymerase chain reaction (PCR) and this was quantified using a NanoDrop® ND-1000 UV–Vis Spectrophotometer (Wilmington, DE, USA) (Ash and Page, 2010).

2.3.3. Detection of the *Van* Genes using PCR

Genes encoding the vancomycin-resistance determinants *vanA* and *vanB* were investigated by PCR using specific primers. Single and multiplex polymerase chain reaction (PCR) were used to screen isolates that were phenotypically resistant to vancomycin (Mac *et al*., 2003, D' Azevedo *et al.,* 2009). The PCR reaction mix (total volume of 20 µl) included 0.2 µM of each 10 pMol primer (see table 1), 1.5 µl of genomic DNA, 4 µl of 5X FIREPOL master mix (containing the following reagents: 1 U FIREPOL Taq HotStart DNA polymerase, 1X PCR Buffer, 200µM of each dNTP (four deoxyribonucleotides: dATP, dCTP, dGTP and dTTP) and 1.5 mM of $MgCl_2$, and sterile deionized water. The primers used in this study are listed in Table 1. PCR amplification was performed using a DNA thermal cycler (Mullis, 1990). The amplification cycles were: initial preheating step at 94º for 2 min; initial denaturation step at 94ºC for 5 min; 30 cycles of amplification (denaturation 94ºC for 1 min, annealing at 54ºC for 1 min, and extension at 72ºC for 2 min); and a final extension at 72ºC for 5 min. The PCR products were electrophoresed through a 1.5 % agarose gel stained with ethidium bromide for 40 min at 100V and photographed under UV light.

Table 1: Primers used in the amplification of resistance genes in isolates of enterococci

Resistance gene/ Determinant	Direction (5'–3')	Primer sequence	Product size (bp)	Reference
vanA	F	CATGAATAGAATAAAAGTTGCAATA	1030	Mac *et al.*, (2003)
	R	CCCCTTTAACGCTAATACGATCAA		
vanA	F	GGGAAAACGACAATTGC	732	D' Azevedo *et al.,* (2009)
	R	GTACAATGCGGCCGTTA		
vanB	F	GTGACAAACCGGAGGCGAGGA	433	Mac *et al.*, (2003)
	R	CCGCCATCCTCCTGCAAAAAA		

2.4. Evaluation of Enterococcal Efflux Activity

The method used was the Ethidium Bromide (EtBr)-Agar Cartwheel method as reported by Martins *et al*., (2013). This method, in conjunction with the use of antibiotic-containing disks, provides an additional advantage for the easy identification and selection of colonies that differ with respect to antibiotic susceptibility and degree of efflux pump activity (Martins *et al*., 2006; 2013).

Two sets of trypticase soy agar (TSA) plates containing EtBr concentrations ranging from 0 to 2.5 mg/l were prepared fresh (same day of the experiment) and kept protected from light. Overnight cultures of the bacterial isolates to be tested were prepared in Nutrient broth and in the following day their concentration adjusted to 0.5 of a McFarland standard. The TSA plates were then divided into twelve sectors by radial lines, forming a cartwheel pattern (Figure 1). The adjusted bacterial cultures were then swabbed on the EtBr-TSA plates starting from the center of the plate to the margin and subsequently incubated at 37ºC for 16 h. After this period, the TSA plates were examined under a U.V. trans-illuminator and the minimum concentration of EtBr that produced fluorescence of the bacterial mass recorded, and the TSA plates photographed. To check for the temperature effect, an additional incubation step was performed. In this case, one of the TSA plate sets was re-incubated at 37ºC, whereas the duplicate set was incubated at 4ºC. At the end of an additional 24 h, the TSA plates were observed and photographed again and the minimal concentration of EtBr that produced fluorescence at each temperature was compared to that evident after the first incubation at 37ºC (Figure 1).

Efflux activity was confirmed by the additional determination of the zones of clearing values for selected antibiotics, known to be efflux pump substrates (tetracycline, ciprofloxacin, gentamicin, vancomycin and oxacillin.), in the presence of the efflux inhibitor (carbonyl cyanide 3-chlorophenylhydrazone - CCCP) which has an inhibitory effect on efflux activity.

Antibiotic susceptibility was tested with the Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates (Bauer *et al*., 1966) as earlier described. Stock solutions of 1mM, 10mM and 20mM of CCCP were prepared by dissolving 1.02, 10 and 20 mg in 5 ml of 95 % ethanol and diluted to make 25, 50 and 100 µg/ml respectively. To monitor its effects on the viability of the organism, 1 mM and 20 mM of CCCP were used with 0.5 ml of each concentration being dispensed into petri dishes containing 20 ml of Mueller-Hinton agar. Gentamicin, ciprofloxacin, tetracycline, vancomycin and oxacillin antibiotic discs were used in the presence of 10 mM CCCP for the confirmation of efflux activity. The inhibition zone diameters were measured using a transparent ruler and correlated into sensitive (S), or resistant (R) based on the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2007).

3. Results and Discussion

The morphological characteristics of the enterococci colonies isolated were observed based on their pattern of growth, color, odor after incubation on the appropriate media. This shows the colonial appearance of the isolates on the media. The microbiologically and biochemically positive strains not identified by sugar fermentation tests were designated as *Enterococcus* spp (Table 2). The thirty-nine (39) antimicrobial resistant enterococci isolates were identified as seventeen (17) *Enterococcus faecium*, six (6) *Enterococcus faecalis* and sixteen (16) *Enterococcus* species (Table 3).

Two different methods of plasmid isolation adopted revealed the absence of plasmids. This study showed the absence of *vanA* and *vanB* in the enterococci isolates from both environmental samples. Isolates from the poultry samples exhibited fifteen (15) resistance patterns while those from the aquaculture samples exhibited 18 resistance patterns indicating a wide variability in the characteristics of the enterococci isolates (Table 4).

The minimal concentration of EtBr that produced fluorescence was significantly greater at 4^0C than at 37^0C, suggesting the presence of an energy-dependent pump (for example, 35% of isolates from aquaculture showed very low fluorescence at 4^0C and at concentrations of 1.5 mg/ L and 2.0 mg/L). Presence of very low fluorescence was seen at concentrations ranging from 1.5 mg/L to 2.5 mg/L of EtBr. *Enterococcus faecium* showed higher frequencies both in aquaculture and poultry than in *Enterococcus faecalis.* Seventeen (17) isolates from aquaculture (7) and poultry (10) (i.e. five (5) *E. faecium*, one (1) *E. faecalis* and four (4) *E.* spp from aquaculture and four (4) *E. faecium*, two (2) *E. faecalis* and four (4) *E.* spp from poultry) showed a negative result when tested with EtBr (Table 5).

After confirmation with carbonyl cyanide 3-chlorophenylhydrazone (CCCP), fourteen (35.89%) isolates expressed active efflux for more than two antimicrobials each, while three (3) and six (6) of these expressed active efflux for all five and four of the antimicrobials tested respectively. Five (5) isolates, four from aquaculture, one (1) from poultry) showed false positive results (fluoresce in the presence of EtBr but when confirmed in the presence of CCCP, showed no increase in the zone of clearing compared to the original result) (Table 6).

Only 24 isolates (61.54 %) were resistant to vancomycin after treatment with CCCP as against 100 % of the isolates before treatment with CCCP. Also 58.97%, 48.72, 48.15 and 28.21 % of the isolates were resistant to ciprofloxacin, oxacillin, tetracycline and gentamicin respectively, after treatment with CCCP. In addition, 28 of the isolates (71.79%) were susceptible to gentamicin, shown by their increased zones of inhibition after treatment with CCCP, while 21 (53.85%), 20 (51.28%), 16 (41.02%) and 15 (38.46%) isolates were susceptible to tetracycline, oxacillin, ciprofloxacin and vancomucin after treatment with CCCP respectively. Vancomycin had the highest frequency of resistance (over expressed efflux activity) in 24 isolates while 11 (28.21%) isolates showed the lowest number of overexpressed efflux activity on gentamicin (Table 7).

Efflux pumps affect all classes of antibiotics, especially the macrolides, aminoglycosides, beta-lactam, tetracyclines and fluoroquinolones because these antibiotics inhibit different aspects of protein and DNA biosynthesis and therefore must be intracellular to exert their effect (Dzidic *et al.,* 2008). Active efflux of fluoroquinolones has also been seen in wild-type strains of *Enterococcus faecalis* and *Enterococcus faecium* (Lynch *et al*., 1997). Among gram-positive bacteria, these organisms are the most resistant to fluoroquinolones (Lynch *et al*., 1997). Also in a similar work carried out by Molale and Bezuidenhout (2016), multiple resistant *Enterococcus* from South African surface water systems were found to harbor efflux pump genes which code for resistance to antibiotics.

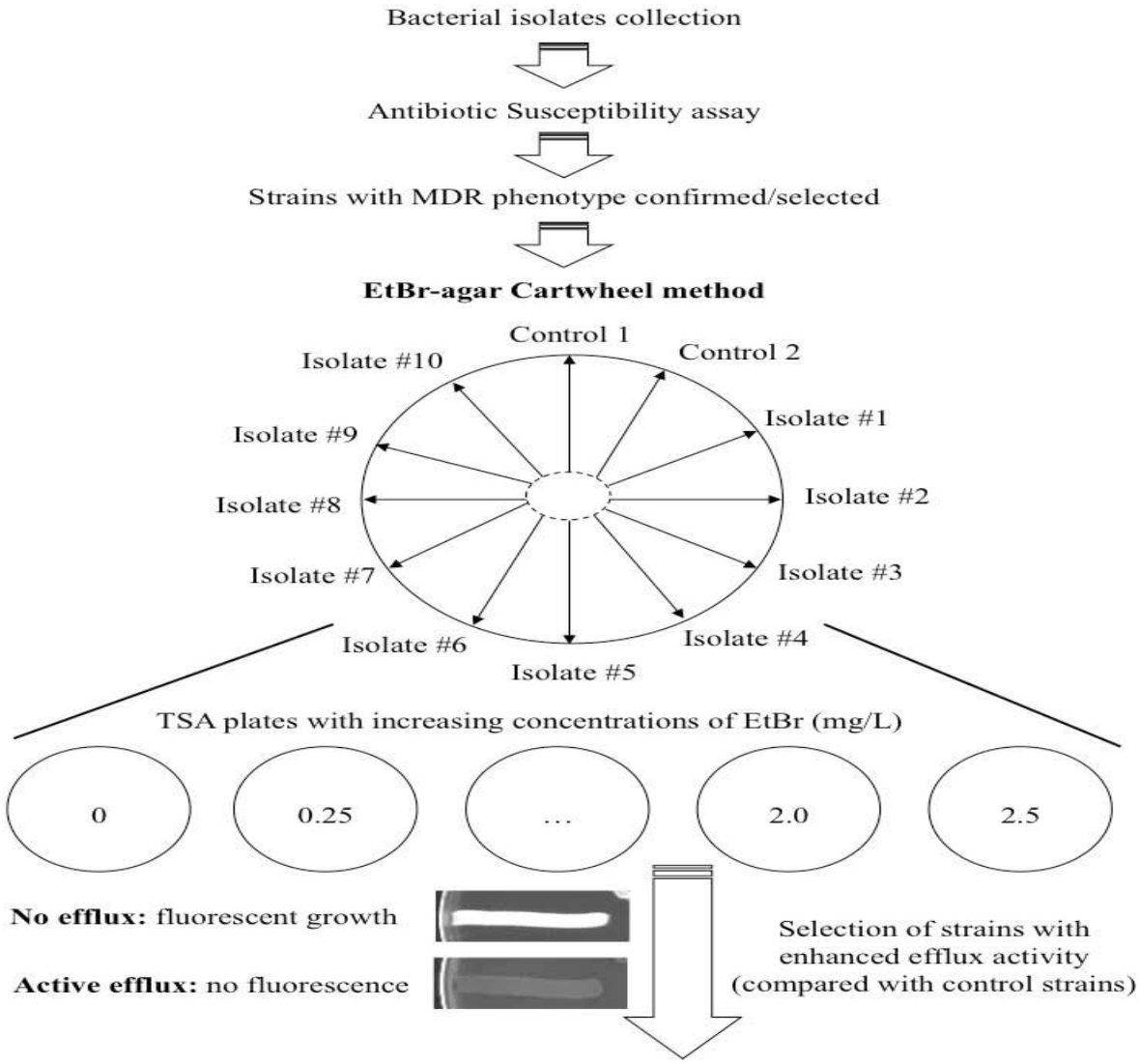

Figure 1: Ethidium Bromide (EtBr)-Agar Cartwheel Method Source: Martins *et al*., (2013)

Confirmation with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) showed a rise in the number of isolates with increased zones of clearing of the antibiotics indicating increased susceptibility of the test isolates, with fewer percentage of the isolates still expressing resistance after exposure to CCCP. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) has an inhibitory effect on efflux activity. This compound confirmed the activity of over-expressed efflux system(s) in the MDR clinical strains identified by this method and this confirmation is important to rule out other factors that may affect fluorescence such as a decrease in the cellular permeability to EtBr (Martins *et al*., 2006). The use of CCCP decreased the resistance level of the isolates. A similar result was also reported by Pasca *et al*. (2005). CCCP could also affect the viability of the microorganisms as reported by Edward *et al*., (2012), who treated isolates with CCCP and discovered complete inhibition of bacterial growth in colonies pretreated with CCCP. Another type of efflux inhibitor, reserpine, was also found to reduce the resistance of clinical *Enterococcus* isolates to flouroquinolones including ciprofloxacin (Jia *et al.,* 2014).

Efflux pumps inhibitors (EPIs) are promising therapeutic agents, as they should restore the activity of standard antibiotics. The efflux pump inhibitor-antibiotic combination is expected to increase the intracellular concentration of antibiotics that are expelled by efflux pumps, decrease the intrinsic bacterial resistance to antibiotics, reverse the acquired resistance associated with efflux pumps overexpression, and reduce the frequency of the emergence of resistant mutant strains (Handzlik *et al*., 2013).

This study indicates a high prevalence of multidrug resistance among enterococci isolated from environmental samples, even in the absence of genes coding for vancomycin resistance. The resistance associated with these environmental isolates was probably due to the presence of active efflux pumps demonstrated by increased susceptibility to test antibiotics, upon exposure of the isolates to an efflux inhibitor. These environmental samples could serve as vehicle to transport these resistant bacteria and genes from animals to humans and become a serious threat to public health. This result can also provide a guide in the development of therapy for the treatment, prevention and control of Enterococcal infections in both humans and animals

Table 2: Biochemical tests and characteristics of identified enterococci

Biochemical Test/ Properties	*Enterococcus faecium*	*Enterococcus faecalis*	*Enterococcus spp.*
Colony color	Reddish-pink	Reddish-brown with metallic sheen	Reddish-brown with metallic sheen
Gram stain	positive (cocci in pairs and chains)	positive (cocci in pairs and chains)	(cocci in pairs and chains)
Catalase	-	-	-
Glucose	+	+	+
Sucrose	+	+	+
Mannitol	+	+	+/-
Maltose	+	+	+
Sorbitol	-	+	+/-
Arabinose	+	-	+/-
Growth at 10^0C	+	+	+
Growth at 45^0C	+	+	+
Alpha hemolysis	+	+	+/-
Motility	-	-	+/-
Yellow pigment	-	-	+/-

Table 3: Frequency of different enterococcal species isolated from poultry and aquaculture sources

Species	No of Isolates (%)		
	Poultry (n=19)	**Aquaculture (n=20)**	**Total (n=39)**
E. faecium	12 (63.16)	5 (25.00)	17 (43.59)
E. faecalis	2 (10.53)	4 (20.00)	6 (15.38)
Enterococcus spp	5 (26.32)	11 (55.00)	16 (41.03)

Table 4: Antimicrobial Resistance Patterns in Enterococci from Poultry and Fishponds

Isolates	Species	Resistance Patterns
Poultry		
1	*E. faecium*	Pn Kn Te Cp Vn
2	,,	Pn Kn Er Te Q/D St Ge Vn Ox
3	,,	Pn Kn Er Te Cp Q/D St Ge Vn Ox
4	,,	Pn Kn Er Te Cp Q/D St Ge Vn Ox
5	,,	Er Te Cp St Vn Ox
6	,,	Pn Er Vn Ox
7	,,	Pn Kn Er Te Cp Q/D St Ge Vn Ox
8	,,	Pn Kn Er Te Cp Q/D Ge Vn Ox
9	,,	Pn Kn Te Cp Q/D St Ge Vn Ox
10	,,	Kn Er Te Cp Q/D St Ge Vn
11	,,	Kn Er Te Cp Q/D St Vn Ox
12	,,	Pn Kn Er St Vn Ox
13	*E. faecalis*	Kn Er Te Cp Q/D Ge Vn Ox
14	,,	Er Te Cp Q/D Vn Ox
15	*E. spp*	Pn K Te Cp Q/D St Vn Ox
16	,,	Pn Kn Er Te Cp Q/D St Ge Vn Ox
17	,,	Te Cp Q/D St Ge Vn
18	,,	Pn Te Cp Q/D St Ge Ox
19	,,	Pn Kn Er Te Cp Q/D St Ge Vn Ox
Isolates	**Species**	**Resistance Patterns**
Fishpond		
A	*E. faecium*	Pn Kn St Vn Ox
B	,,	Pn Kn Er Te Cp Q/D St Ge Vn Ox
C	,,	Cp Q/D Vn
D	,,	Te Cp Ge Vn Ox
E	,,	Pn Er Te St Ge Vn Ox
F	*E. faecalis*	Pn Kn Er Te Cp Q/D St Ge Vn Ox
G	,,	Er Q/D St Vn Ox
H	,,	Pn Kn Te Cp Q/D Vn
I	,,	Pn Er Te Cp Q/D St Ge Vn Ox
J	,,	Pn Er Vn Ox
K	*E. spp*	Kn Er Cp Q/D Vn Ox
L	,,	Vn Ox
M	,,	Pn Er Te Cp Q/D St Vn
N	,,	Pn Kn Cp Q/D Ge Vn
O	,,	Pn Kn Er Te Cp Q/D St Ge Vn Ox
P	,,	Pn Kn Er Te Cp Q/D St Ge Vn Ox
Q	,,	Pn Kn Er Te Q/D St Ge Vn Ox
R	,,	Pn Cp St Vn Ox
S	,,	Kn Er Te Cp St Ge Vn Ox
T	,,	Vn

Key: Pn-Penicillin G; Kn-Kanamycin; Er-Erythromycin; Te-Tetracycline; Q/D-Quinupristine/ Dalfopristine; Ge-Gentamicin; St-Streptomycin; Vn-Vancomycin; Cp-Ciprofloxacin; Ox-Oxacillin.

Table 5: Frequency of Isolates that showed fluorescence in the presence of Ethidium Bromide (EtBr)

Temperature (°C)	Isolate	No of fluorescent isolates (%) Source of isolate: Aquaculture EtBr conc. (mg/l) 1.5	2.0	2.5	Poultry EtBr conc. (mg/l) 1.5	2.0	2.5
	E. faecium	**(n=5)**			**(n=12)**		
4		2(40.0)	1 (20.0)	2(40.0)	2 (16.7)	5 (41.7)	6 (50.0)
37		0(0.0)	1 (20.0)	1 (20.0)	2 (16.7)	2 (16.7)	3 (25.0)
	E. faecalis	**(n=4)**			**(n=2)**		
4		3 (75.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
37		0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	E. sp	**(n=11)**			**(n=5)**		
4		2 (18.2)	5 (45.5)	2 (18.2)	0(0.0)	0(0.0)	1 (20.0)
37		0(0.0)	1 (9.1)	2 (18.2)	0(0.0)	1 (20.0)	1 (20.0)
	Total	**Aquaculture (n=20)**			**Poultry (n=19)**		
4		7 (35.0)	7 (35.0)	4 (20.0	2 (10.5)	5 (26.3)	4 (21.1)
37		0(0.0)	2 (10.0)	3 (15.0)	2 (10.5)	3 (15.8)	7 (36.8)

Table 7: Susceptibility of test enterococci isolates to antibiotics before and after treatment with CCCP

Antibiotics	No of resistant enterococci isolates (%)					
	Before treatment with CCCP			After treatment with CCCP		
	Poultry (n=19)	Aquaculture (n=20)	Total (n=39)	Poultry (n=19)	Aquaculture (n=20)	Total (n=39)
Vancomycin	19 (100)	20 (100)	39 (100.0)	12(63.16)	12 (60.0)	24 (61.54)
Ofloxacin	16 (84.21)	13 (65.0)	29(74.36)	15 (78.95)	8 (40.0)	23 (58.97)
Oxacillin	16 (84.21)	14 (70.0)	30 (76.92)	9 (47.37)	10 (50.0)	19 (48.72)
Tetracycline	17 (89.47)	12 (60.0)	29 (74.36)	13 (68.42)	5 (25.0)	18 (48.15)
Gentamicin	12 (63.16)	11 (55.0)	23 (58.97)	7 (36.84)	4 (20.0)	11 (28.21)

Table 6: Confirmation of efflux activity with CCCP and antibiotics

Isolate	Source	Conc. of EtBr (mg/L)						Confirmation test with CCCP and antibiotics					Remark
		1.5		2.0		2.5							
		37	4	37	4	37	4	Van	Tet	Cip	Gen	Oxa	
A	Aquaculture	-	-	-	+	-	-	-	-	-	-	-	false positive
B	"	-	+	+	+	+	+	+	+	+	+	-	efflux system on van, tet, cip, &gen
E	"	-	+	-	-	-	+	-	-	-	-	-	false positive
F	"	-	+	-	-	-	-	+	+	-	+	-	efflux system on van, tet, &gen
G	"	-	+	-	-	-	-	-	-	-	-	-	false positive
I	"	-	+	-	-	-	-	+	+	-	+	-	efflux system on van, tet, &gen
J	"	-	+	-	+	-	-	+	+	+	+	+	efflux system on van, tet, cip, gen & oxa
K	"	-	-	-	+	-	+	+	+	+	+	+	efflux system on van, tet, cip, gen & oxa
L	"	-	-	-	+	-	+	+	-	-	-	+	efflux system on van & oxa
M	"	-	+	-	-	-	-	+	+	+	+	-	efflux system on van, tet, cip, &gen
N	"	-	-	-	+	+	-	+	+	+	+	-	efflux system on van, tet, cip, &gen
R	"	-	-	+	+	-	-	-	-	-	-	+	efflux system on oxa
T	"	-	-	-	-	+	-	-	-	-	-	-	false positive
2	Poultry	-	-	-	+	-	+	+	+	-	+	+	efflux system on van, tet, gen & oxa
4	"	-	-	-	+	-	+	+	+	-	+	+	efflux system on van, tet, gen & oxa
5	"	-	-	-	+	-	-	-	-	-	-	-	false positive
6	"	+	+	+	+	-	+	+	-	-	-	+	efflux system on van & oxa
7	"	+	+	+	+	+	-	+	-	-	+	+	efflux system on van, gen & oxa
8	"	-	-	-	-	+	-	+	+	-	+	+	efflux system on van, tet, gen & oxa
11	"	-	-	-	-	+	+	+	-	-	-	-	efflux system on van
12	"	-	-	-	-	+	+	-	-	-	-	+	efflux system on oxa
16	"	-	-	+	-	+	+	+	+	+	+	+	efflux system on van, tet, cip, gen & oxa

Key: Tet-Tetracycline; Gen-Gentamicin; Van-Vancomycin; Cip-Ciprofloxacin; Oxa-Oxacillin; CCCP-Carbonyl cyanide 3-chlorophenylhydrazone; EtBr- Ethidium Bromide

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