https://dx.doi.org/10.4314/ijs.v22i3.4

DIVERSITY, ANTIMICROBIAL CHARACTERIZATION AND BIOFILM FORMATION OF ENTEROCOCCI ISOLATED FROM AQUACULTURE AND SLAUGHTERHOUSE SOURCES IN BENIN CITY, NIGERIA

[1,2*]Igbinosa, E. O., [1,3]Beshiru, A. and [4]Odjadjare, E.E.O.

[1]Applied Microbial Processes & Environmental Health Research Group (AMPEHREG), Department of Microbiology, Faculty of Life Sciences, University of Benin, Private Mail Bag 1154 Benin City 300001, Nigeria.

[2]Sustainable Development Office, University of Benin, Private Mail Bag 1154, Benin City 300283, Nigeria.

[3]Department of Microbiology, College of Natural and Applied Sciences, Western Delta University, Private Mail Bag 10 Oghara 331101, Delta State, Nigeria.

[4]Environmental, Public Health and Bioresource Microbiology Research Group (EPHBIOMREG), Department of Biological Sciences, Benson Idahosa University, Private Mail Bag 1100, Benin City 300001, Nigeria.

*Corresponding author's e-mail: eigbinosa@gmail.com

(Received: 9th January, 2020; Accepted: 7th August, 2020)

ABSTRACT

The present study was designed to characterize Enterococci isolates obtained from water samples at aquaculture and slaughterhouse facilities in Benin City, Nigeria. A total of 144 water samples were collected from aquaculture and slaughterhouse facilities. All samples were analyzed using classical microbiological and molecular-based methods. Enterococci were identified using specific primer sets (genus and species specific primers) and are as follows: *E. faecalis* 36 (25.5%); *E. faecium* 39 (27.7%); *E. durans* 19 (13.4%); *E. casseliflavus* 13 (9.2%); *E. hirae* 14 (9.9%) and other *Enterococcus* species 20 (14.2%). The resistance profile of the bacterial strains to antibiotics was as follows: [tetracycline (n=67, 47%)]; [vancomycin (n=74, 52%)]; [erythromycin (n=91, 64%)] and [penicillin (n=141, 100%)]. Enterococci virulence genes detected include: [*gel*E (n=120, 85.1%)]; [*cyl*A (n= 52, 36.9%)]; [*hyl* (n=96, 68.1%)]; [*esp* (n=135, 95.8%)]; [*ace* (n= 127, 90.1%)] and [*agg* n=118, 83.7%)]. Antibiotic-resistant gene detected from the phenotypic resistant isolates were 55/74 (74.3%) *van*A; 61/67 (91.1%) *tet*C; 122/141 (86.5%) *bla*pse1 and 62/91 (68.1%) *erm*A. Antibiotic-resistant coupled with biofilm formation potential of *Enterococcus* species include penicillin+biofilm 116 (82.3%); erythromycin+biofilm 85 (60.3%); and vancomycin+biofilm 74 (52.3%). Findings from this study reveal that strains with the ability of forming biofilms have enhanced antimicrobial resistance. Continuous monitoring of slaughterhouses and aquaculture facilities is necessary to guarantee food safety.

Key Words: Aquaculture, Biofilm, *Enterococcus*, Environments, Resistance, Slaughterhouse

INTRODUCTION

Enterococci have the capacity to thrive in harsh environmental conditions and enables them to proliferate from low-density commensals to a dominant population of microbiota; thus forming a predisposition for pathogenesis (Staley *et al*., 2014). Regardless of being a predominant adherent of the human intestinal flora, they are no longer classified as "Generally Recognized As Safe" (GRAS) organisms (Rathnayake *et al*., 2012); as some of its members have been reported as major causes of hepatobiliary sepsis, urinary tract infections, surgical wound infections, endocarditis, neonatal sepsis, bacteraemia and other nosocomial infections (Elhani *et al*., 2014). Enterococci, being a carrier of a multiplicity of mobile genetic elements are responsible for diverse host range gene acquisition or gene dissemination which jointly makes them resist multiple antibiotics (Beshiru *et al*., 2017). Enterococci are efficient at being resistant to antibiotics, revealing a variety of pathway for intrinsic and acquired resistance. The organisms have been reported to have incredible genome plasticity with a capacity to utilize transposons, plasmids and insertion sequences to proficiently acquire and spread mobile resistance determinants, and consequently expediting the spread of resistance elements (Cattoir and Leclercq, 2013). Antimicrobial resistance is a global phenomenon intertwined with increased illness and death (Frieri *et al*., 2017). Multidrug-resistance in Gram-positive bacteria has led to difficulty treating infections associated with the application of commonly used antibiotics. The significant upsurge in emerging antibiotics resistance across the globe, coupled with dilapidating infection control infrastructure, has resulted in the easy spread of antibiotic-resistant bacteria to other environments and patients

(Chajecka-Wierzchowska *et al*., 2016). Increased antibiotic resistance encountered in clinical bacterial pathogens, commensals, bacteria strains of environmental origin, bacteriophages and mobile genetic elements, act as a reservoir of antibiotic resistance genes (ARGs) through which bacterial pathogens can acquire and disseminate resistance via horizontal gene transfer (HGT) (von Wintersdorff *et al*., 2016).

Traditionally, 90% of enterococcal infections originate from *E. faecalis* with just 10% attributed to *E. faecium*. However, the frequency of *E. faecium* has over the years increased to 40% (Daniel *et al*., 2015). Other species of enterococci, such as *E. casseliflavus*, *E. avium, E. cecorum, E. durans, E. dispar, E. gallinarum, E. malodoratus, E. hirae, E. mundtii, E. raffinosus, E. pseudoavium, E. saccharolyticus, E. solitarius* and *E. seriolicida*, are primarily found in the gastrointestinal tracts of several animals but are rarely recovered from human infections (Daniel *et al*., 2015).

Studies relating to the spread of enterococci from food-producing animals to humans have concentrated on disease-causing agents that pose a threat to human health (Marshall and Levy, 2011). Due to the importance of *Enterococcus* spp. to the farming industry, food security and public health, additional information on the transmission and genetics of antibiotic-resistant enterococci are imperative. Regulations and legislation to manage the use and supply of antimicrobials are very deficient in developing countries (FAO, 2012). The occurrence of multiple drug-resistant (MDR) pathogenic enterococci has increased rapidly (Kang and Song, 2013).

Slaughterhouses are facilities designed and licensed for receiving, holding, slaughtering, inspecting meat from animals and meat products before releasing to the public. Aquaculture environs are also regarded as facilities where fish are nurtured from fingerlings to maturation. The discharge of wastewater especially from aquaculture and slaughterhouses into the environment (water and soil) has increased tremendously due to the persistent demand for meat and fish production to meet the growing need of consumers. The meat and fish processing industry produce a lot of wastewater due to slaughtering of animals, change of water in fish ponds, cleaning of slaughterhouse facilities etc.

In this study, we evaluated the effluent quality of aquaculture and slaughterhouse with regards to the presence of antibiotic resistance enterococci, antibiotic resistance genes (ARGs), and biofilm-forming enterococci. The findings of this study will be vital for improving suitable effluent management interventions. This will help to advance the ideals of the United Nations Sustainable Development Goal 3 (good health and well-being) and Goal 6 (clean water and sanitation) framework in developing countries such as Nigeria.

MATERIALS AND METHODS

Sample Collection

A total of 144 samples comprising 72 samples each from aquaculture and slaughterhouse water sources in Benin City Nigeria, were collected using sterile plastic containers between the months of April and November 2017. The specific locations and names of the slaughterhouse and aquaculture environments were withheld as part of the consent by the respective slaughterhouse and aquaculture heads prior to sampling. During sampling from both environments, the water samples were used to rinse the plastic containers three times before collection. Samples include the water source (borehole to storage tank), the point of usage (fish pond and slaughter site), and point of discharge (drains and other open discharge points). The samples were transported in cooling boxes containing ice packs to the Applied Microbial Processes & Environmental Health Research Group (AMPEHREG) Laboratory for analysis within 4 h of sample collection.

Isolation of *Enterococcus* species from the Samples

Samples from the point of discharge and point of usage were serially diluted (10^{1}-10^{8}). From the diluents, an aliquot of 100 µl was spread-plated on bile aesculin azide (BAA) agar (Merck, Germany) and nutrient agar (Lab M, United Kingdom), while the same aliquot was also spread-plated directly from the source water samples without dilution. All plates were incubated for 18-24 h at 37 °C. Colonies on both BAA agar and nutrient agar were enumerated and expressed in colony-forming unit per millilitre (cfu/ml). Thereafter, an average of

two to three black hallow colonies from BAA agar was sub-cultured on fresh BAA agar and incubated for another 18-24 h at 37 °C. Isolates were then purified on nutrient agar for another 18-24 h at 37 °C. Purified isolates were stored on tryptone soy broth (Merck, Darmstadt, Germany) with 30% glycerol at -20 °C until needed for further analysis.

Identification of the *Enterococcus* species

All Gram-positive cocci, oxidase- negative (using oxidase strip), catalase-negative, black hallow colonies on BAA agar were subjected to biochemical characterization using Analytical Profile Index (API) 20E (BioMerieux, Marcy-l'Étoile, France) following the manufacturer's instruction. *Enterococcus faecium* (ATCC 19434) was used as positive control strain; while *Staphylococcus aureus* (ATCC 12600) was used as a negative control. The API strips were critically studied and identities of the isolates secured using API lab plus software (BioMerieux, Marcy l'Etoile, France).

DNA Extraction and Molecular Screening of *Enterococcus* Identity

The DNA extraction was carried out using the heat-treated method. Polymerase chain reaction (PCR) using genus-specific and species-specific primers and PCR conditions previously described were used in the identification of the *Enterococcus* species (Igbinosa and Beshiru, 2019). The Peltier-based Thermal Cycler (MG96þ/Y, Zhejiang China) was used in the amplification process. Electrophoresis of the PCR amplicons was performed with 1.0% agarose gel (CLS-AG100, Warwickshire, United Kingdom) in 0.5× TAE buffer (40 mM Tris-HCl, pH 8.5, 1 mM EDTA, 20 mM sodium acetate) and allowed to run for 55 mins at 100 V. The gels were visualized under a UV transilluminator (EBOX VX5, Vilber Lourmat, France).

Antimicrobial Susceptibility Profile of the Enterococci Isolates

Enterococcus species were further subjected to antimicrobial susceptibility screening using the disc diffusion method (Kirby-Bauer). Twelve antibiotics were used in this study belonging to nine (9) groups of antimicrobials. They include: Glycopeptide (vancomycin 30 µg), Tetracyclines (tetracycline 30 µg), Fluoroquinolone (ciprofloxacin 50 µg), Carbapenems (meropenem 10 µg, imipenem 10 µg), Lincosamides (clindamycin 2 µg), Aminoglycosides (kanamycin 30 µg, streptomycin 10 µg), Macrolides (erythromycin 15 µg), Phenicols (chloramphenicol 30 µg), and Penicillin (penicillin G 10 µg, and piperacillin 100 µg). Purified colonies were re-suspended on normal saline to obtain 0.5 McFarland turbidity standards. Using a sterile swab dipped in the suspension and the excess liquid removed by pressing against the wall inside the test tubes; the entire surface of Mueller Hinton agar plates (Merck Germany) was inoculated with the test isolates. The antibiotics disc were aseptically impregnated equidistance from each other with an average of 6 antibiotics disc per plate. The plates were allowed to stand for 15 min before incubation at 37 °C for 18-24 h. Zones of inhibition were measured and interpreted using breakpoints from the Clinical and Laboratory Standard Institute (2017).

Phenotypic Virulence

The phenotypic virulence profile of the isolates was determined as described by Beshiru and Igbinosa (2018). Colonies cultivated on tryptone soy agar (TSA) (Merck, Darmstadt, Germany) were re-suspended in 20 ml of tryptone soy broth (TSB). The turbidity of this suspension was adjusted to 10^6 cells/ml using the McFarland standard. Haemolytic activity was determined on a sheep blood agar plate. Lipase activity was elucidated on TSA. Gelatinase production was determined on gelatin medium. DNA degrading activity ascertained on DNase agar plates. The presence of surface-layer (Slayer) was assessed by streaking cultures on TSA plates, enhanced with 0.1 mg/ml Coomassie brilliant blue R 250 (Merck, Darmstadt Germany). All experiments were performed in triplicates.

Characterization of the Biofilm

Biofilm formation for *Enterococcus* species was quantitatively assessed using the microtitre plate method. Ninety-six (96) wells microtiter plates were dispensed with 200 µl of nutrient broth (Lab M, Lancashire, United Kingdom) and thereafter inoculated with 20 µl of enterococci isolates cultivated overnight and re-standardized to 0.5 McFarland turbidity standards and incubated

overnight for 18-24 h. Constituents of respective wells were removed and plates rinsed with sterile phosphate-buffered saline (PBS) and allowed to air dry and thereafter stained with 200 µl of 1% crystal violet for 30 min. Respective wells were rinsed with de-ionised water to remove the crystal violet and allowed to dry at 28±2 °C. Crystal violet dye bound to adherent cells was made soluble via 150 µl of absolute ethanol. The optical densities (OD) of the plates were determined at a wavelength of 570 nm with the aid of a Microplate reader (Synergy MxBiotekR USA). Mean OD of each triplicate result was calculated along with negative and positive controls. Isolates were grouped as strong (ODi> 0.12), moderate (ODi = 0.1 < 0.12), weak (ODc<ODi< 0.1) and non-biofilm producer (ODi<ODc) in accordance with the modified methods of Igbinosa and Beshiru, (2019).

PCR screening of *Enterococcus* species for Antibiotic Resistance and Virulence Genes

Enterococcus species confirmed using genus and species-specific primers in this study were further screened for their capacity to harbour antimicrobial resistance and virulence genes. The various virulence signatures screened included *gel*E, *cyl*A, *agg*, *esp*, *ace* and *hyl* genes (Igbinosa and Beshiru, 2019); while the antibiotic resistance genes screened included the *van*A, *van*B, *van*C21/2, tetA, tetB, *tet*C gene, *erm*A, *erm*B, *erm*C, bla_{TEM} gene and *bla*pse1 using specific primers and PCR conditions (Igbinosa and Beshiru, 2019).

Statistical Analysis

Statistical analysis was carried out on the data using the Statistical Package (SPSS) version 21.0 and Microsoft Excel 2013. Mean values were expressed in mean and standard deviation using descriptive statistics. Correlation analysis was carried out to determine the effect of the phenotypic virulence variable on similar genotypic virulence variable. P-values less than 0.05 (p<0.05) were considered statistically significant.

RESULTS

The Mean Population Cell Density

The mean heterotrophic bacteria count from the aquaculture point of discharge was 4.1 ± 0.02 $\times 10^9$ cfu/ml while that of the slaughterhouse was 1.1 ± 0.51 $\times 10^{10}$ cfu/ml (Table 1). The mean enterococci count from the aquaculture point of discharge was 3.9 ± 0.10$\times 10^4$ cfu/ml while that of the slaughterhouse was 2.1 ± 0.02 $\times 10^5$ cfu/ml.

Table 1: The Mean Population Cell Density of Heterotrophic Bacteria and Enterococci Isolates from Aquaculture and Slaughterhouse Environments

Sampling point	Target bacteria	Aquaculture environment			Slaughterhouse environment		
		Minimum (cfu/ml)	Maximum (cfu/ml)	Mean ± SD (cfu/ml)	Minimum (cfu/ml)	Maximum (cfu/ml)	Mean ± SD (cfu/ml)
Water source	Heterotrophic bacteria count	2×10^0	2.1×10^1	$1.2 \pm 0.10 \times10^1$	3.0×10^0	1.9×10^1	$1.0 \pm 0.05 \times10^1$
	Enterococci count	0×10^0	3.0×10^0	$2.0 \pm 0.01 \times10^0$	0×10^0	5.0×10^0	$2.2 \pm 0.01 \times10^0$
Point of usage	Heterotrophic bacteria count	1.5×10^5	2.3×10^7	$2.1 \pm 0.01 \times10^6$	6.6×10^6	4.2×10^8	$3.8 \pm 0.00 \times10^7$
	Enterococci count	1.9×10^2	1.0×10^3	$1.7 \pm 0.03 \times10^2$	1.9×10^3	1.0×10^5	$4.5 \pm 0.03 \times10^4$
Point of discharge	Heterotrophic bacteria count	6.2×10^8	2.7×10^{10}	$4.1 \pm 0.02 \times10^9$	3.5×10^8	2.5×10^{11}	$1.1 \pm 0.51 \times10^{10}$
	Enterococci count	3.0×10^3	2.8×10^5	$3.9 \pm 0.10 \times10^4$	6.5×10^3	2.2×10^6	$2.1 \pm 0.02 \times10^5$

Distribution of *Enterococcus* species

The aquaculture environments: 9/67 (13.4%) enterococci were recovered from the water source, 27/67 (40.2%) from the point of usage and 31/67 (46%) from the point of discharge. From the slaughterhouse environments, 11/74 (14.8%) enterococci were recovered from the water source, 24/74 (32.4%) from the point of usage and 39/74 (52.7%) from the point of discharge (Table 2). Overall, *E. faecalis* 36/141

(25.5%), *E. faecium* 39/141 (27.7%), *E. durans* 19/141 (13.4%), *E. casseliflavus* 13/141 (9.2%), *E. hirae* 14/141 (9.9%), and other *Enterococcus* species 20/141 (14.2%) were recovered from both aquaculture and slaughterhouse environments using genus and species-specific primers (Table 2).

Table 2: Distribution of *Enterococcus* species in Aquaculture and Slaughterhouse Environments

Enterococcus species	Aquaculture environment *n*=67			Slaughterhouse environment *n*=74			Total *n*= 141
	Water source *n*=9	Point of usage *n*=27	Point of discharge *n*=31	Water source *n*=11	Point of usage *n*=24	Point of discharge *n*=39	
E. faecalis	1 (11.1)	7 (25.9)	13 (41.9)	0 (0)	5 (20.8)	10 (25.6)	36 (25.5)
E. faecium	3 (33.3)	12 (44.4)	8 (25.8)	2 (18.2)	6 (25)	8 (20.5)	39 (27.7)
E. durans	1 (11.1)	5 (18.5)	4 (12.9)	1 (9.1)	4 (16.7)	4 (10.3)	19 (13.4)
E. casseliflavus	1 (11.1)	0 (0)	5 (16.1)	1 (9.1)	2 (8.3)	4 (10.3)	13 (9.2)
E. hirae	0 (0)	1 (3.7)	0 (0)	2 (18.2)	5 (20.8)	6 (15.4)	14 (9.9)
Other *Enterococcus* species	3 (33.3)	2 (7.4)	1 (3.2)	5 (45.4)	2 (8.3)	7 (17.9)	20 (14.2)

Antibiotics resistance profile of the *Enterococcus* species

The results of resistant profile of *Enterococcus* species in table 3 in this study include vancomycin 74 (52%), tetracycline 67 (47%), clindamycin 131 (93%), streptomycin 104 (73%), chloramphenicol 141 (100%) and penicillin G 141 (100%). It was observed that some strains of *E. faecalis* 8/36 (22%), *E. faecium* 7/39 (18%), *E. durans* 1/19 (5%), *E. casseliflavus* 2/13 (15%), *E. hirae* 1/14 (7%) and other *Enterococcus* species 2/20 (10%) were resistant to all the antibiotics used in this study (Table 4). More so, *E. faecalis* 34/36 (94%), *E. faecium* 35/39 (89%), *E. durans* 17/19 (89%), *E. casseliflavus* 13/13 (100%), *E. hirae* 12/14 (85%) and other *Enterococcus* species 17/20 (85%) were resistant to CLI^R, CHL^R, PEN^R (Table 4).

Table 3: Antimicrobial Profile of *Enterococcus* species Isolated during this Study

Antibiotics	Antibiogram Profile																	
	E. faecalis n = 36			*E. faecium n* = 39			*E. durans n* = 19			*E. casseliflavus n* = 13			*E. hirae n* =14			Other Enterococcus species *n* = 20		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
VAN	23 (63)	4 (11)	9 (25)	26 (66)	3 (7)	10 (25)	6 (32)	8 (42)	5 (26)	5 (38)	5 (38)	3 (23)	6 (42)	6 (42)	2 (11)	8 (40)	12 (60)	0 (0)
TET	21 (58)	15 (41)	0 (0)	18 (46)	16 (41)	5 (13)	10 (52)	1 (5)	8 (42)	4 (30)	2 (15)	7 (53)	5 (35)	0 (0)	9 (64)	9 (45)	3 (15)	8 (40)
CIP	9 (25)	9 (25)	18 (50)	11 (28)	7 (17)	21 (53)	3 (15)	4 (21)	12 (63)	4 (30)	6 (46)	3 (23)	3 (21)	5 (35)	6 (42)	4 (20)	1 (5)	15 (75)
MEM	11 (30)	14 (38)	11 (30)	15 (38)	16 (41)	8 (20)	7 (36)	10 (52)	2 (10)	5 (38)	8 (61)	0 (0)	1 (7)	4 (28)	9 (64)	3 (15)	3 (15)	14 (70)
IMI	20 (55)	10 (27)	6 (16)	17 (43)	9 (23)	13 (33)	5 (26)	9 (47)	5 (26)	7 (53)	6 (46)	0 (0)	4 (28)	8 (57)	2 (11)	6 (30)	8 (40)	6 (30)
CLI	35 (97)	1 (3)	0 (0)	36 (92)	3 (7)	0 (0)	17 (89)	2 (10)	0 (0)	13 (100)	0 (0)	0 (0)	12 (85)	2 (11)	0 (0)	18 (90)	2 (10)	0 (0)
KAN	13 (36)	20 (55)	3 (8)	15 (38)	22 (56)	2 (5)	3 (15)	14 (73)	2 (10)	5 (38)	6 (46)	2 (15)	3 (21)	10 (71)	1 (7)	3 (15)	3 (15)	14 (70)
STR	33 (91)	3 (8)	0 (0)	37 (94)	0 (0)	2 (5)	16 (84)	2 (10)	1 (5)	11 (84)	1 (7)	1 (7)	2 (11)	11 (78)	1 (7)	5 (25)	9 (45)	6 (30)
ERY	29 (80)	6 (16)	1 (3)	32 (82)	7 (18)	0 (0)	6 (32)	12 (63)	1 (5)	10 (76)	3 (23)	0 (0)	5 (35)	0 (0)	9 (64)	9 (45)	2 (10)	9 (45)
CHL	36 (100)	0 (0)	0 (0)	39 (100)	0 (0)	0 (0)	19 (100)	0 (0)	0 (0)	13 (100)	0 (0)	0 (0)	14 (100)	0 (0)	0 (0)	20 (100)	0 (0)	0 (0)
PIP	12 (33)	11 (30)	13 (36)	11 (28)	16 (41)	12 (30)	1 (5)	3 (15)	15 (78)	2 (15)	8 (61)	3 (23)	1 (7)	5 (35)	8 (57)	2 (10)	5 (25)	13 (65)
PEN	36 (100)	0 (0)	0 (0)	39 (100)	0 (0)	0 (0)	19 (100)	0 (0)	0 (0)	13 (100)	0 (0)	0 (0)	14 (100)	0 (0)	0 (0)	20 (100)	0 (0)	0 (0)

Legend: VAN: Vancomycin (30 µg); TET: Tetracycline (30 µg); CIP: Ciprofloxacin (50 µg); MEM: Meropenem (10 µg); IMI: Imipenem (10 µg), CLI: Clindamycin (2 µg), KAN: Kanamycin (30 µg); STR: Streptomycin (10 µg); ERY: Erythromycin (15 µg); CHL: Chloramphenicol (30 µg), PEN: Penicillin G (10 µg), and PIP: Piperacillin (100 µg). Values in parenthesis represent percentage (%)

Table 4: Multidrug Resistant Profile of *Enterococcus* species Isolated during this Study

Number of antimicrobial class	Number of antibiotics	Resistance phenotype	*E. faecalis* **n=36**	*E. faecium* **n=39**	*E. durans* **n=19**	*E. casseliflavus* **n=13**	*E. hirae* **n=14**	**Other *Enterococcus* species n=20**
9	12	VAN^R, TET^R, CIP^R, MEM^R, IMI^R, CLI^R, KAN^R, STR^R, ERY^R, CHL^R, PIP^R, PEN^R	8 (22)	7 (18)	1 (5)	2 (15)	1 (7)	2 (10)
8	11	VAN^R, TET^R, MEM^R, IMI^R, CLI^R, KAN^R, STR^R, ERY^R, CHL^R, PIP^R, PEN^R	11 (30)	10 (25)	1 (5)	2 (15)	1 (7)	2 (10)
8	10	VAN^R, TET^R, MEM^R, IMI^R, CLI^R, KAN^R, STR^R, ERY^R, CHL^R, PEN^R	11 (30)	14 (35)	3 (15)	4 (30)	1 (7)	3 (15)
8	9	VAN^R, TET^R, IMI^R, CLI^R, KAN^R, STR^R, ERY^R, CHL^R, PEN^R	12 (33)	14 (35)	3 (15)	4 (30)	2 (14)	3 (15)
8	8	VAN^R, TET^R, IMI^R, CLI^R, STR^R, ERY^R, CHL^R, PEN^R	17 (47)	15 (38)	5 (26)	4 (30)	2 (14)	4 (20)
7	7	VAN^R, TET^R, CLI^R, STR^R, ERY^R, CHL^R, PEN^R	20 (55)	16 (41)	5 (26)	4 (30)	2 (14)	5 (25)
6	6	VAN^R, TET^R, CLI^R, ERY^R, CHL^R, PEN^R	20 (55)	17 (43)	5 (26)	4 (30)	4 (28)	8 (40)
5	5	TET^R, CLI^R, ERY^R, CHL^R, PEN^R	20 (55)	18 (46)	6 (31)	4 (30)	5 (35)	9 (45)
4	4	CLI^R, ERY^R, CHL^R, PEN^R	27 (75)	30 (77)	6 (31)	9 (69)	5 (35)	9 (45)
3	3	CLI^R, CHL^R, PEN^R	34 (94)	35 (89)	17 (89)	13 (100)	12 (85)	17 (85)

Legend: VAN: Vancomycin (30 µg); TET: Tetracycline (30 µg); CIP: Ciprofloxacin (50 µg); MEM: Meropenem (10 µg); IMI: Imipenem (10 µg), CLI: Clindamycin (2 µg), KAN: Kanamycin (30 µg); STR: Streptomycin (10 µg); ERY: Erythromycin (15 µg); CHL: Chloramphenicol (30 µg), PEN: Penicillin G (10 µg), and PIP: Piperacillin (100 µg). Values in parenthesis represent percentage (%).

Distribution of Phenotypic Virulence factors in the *Enterococcus* species

The distribution of virulence factors in the *Enterococcus* species revealed gelatinase activity: [*E. faecalis* 33 (91.7%), *E. faecium* 38 (97.4%), *E. durans* 16 (84.2%), *E. casseliflavus* 12 (92.3%), *E. hirae* 7 (50%) and other *Enterococcus* species 16 (80%)]; beta-haemolysis [*E. faecalis* 16 (44.4%), *E. faecium* 20 (51.3%), *E. durans* 5 (26.3%), *E. casseliflavus* 8 (61.5%), *E. hirae* 5 (35.7%) and other *Enterococcus* species 4 (20%)]; and S-layer formation (all *Enterococcus* species 141 (100%). Beta-haemolysis occurred in 58 (41.1%) isolates while gelatinase activity occurred in 122 (86.5%) of the enterococci isolates. None of the isolates showed DNA degrading activity (Figure 1).

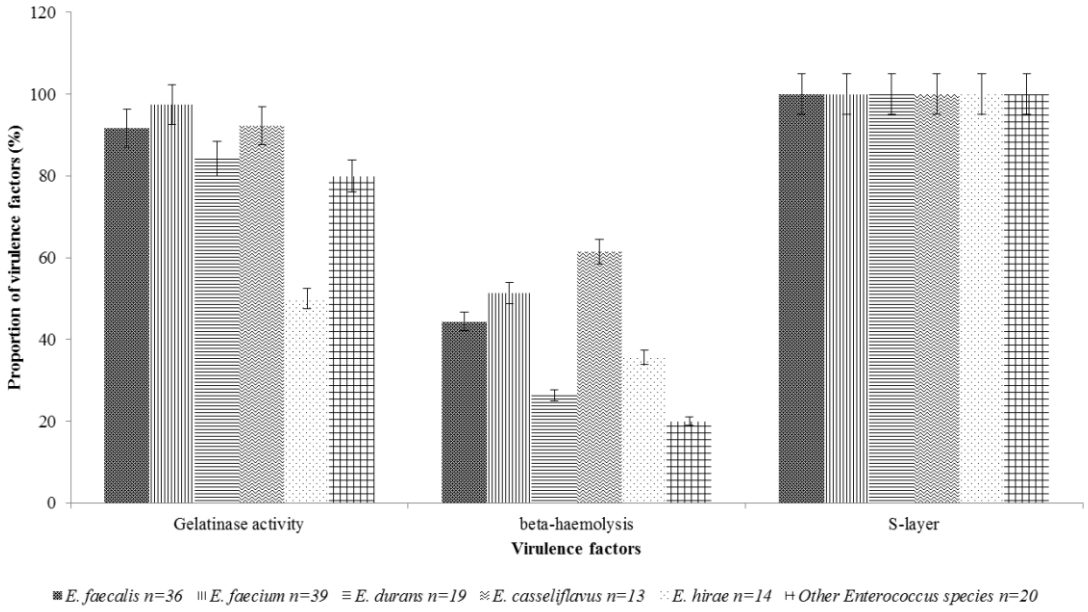

Figure 1: Distribution of Virulence Factors in the *Enterococcus* species

Distribution of Antibiotic Resistance, Virulence genes and Biofilm Formation

The distribution of antibiotic resistance genes in the *Enterococcus* species revealed the presence of *van*A 55/74 (74.3%), *van*B 50/74 (67.6%), *van*C 21/2 4/74 (5.4%), *tet*A 50/67 (74.6%), *tet*C 61/67 (91.1%), bla_{TEM} 110/141 (78%), *bla*pse1 122/141 (86.5%), *erm*A 62/91 (68.1%), *erm*B 55/91 (60.4%), and *erm*C 5/91 (5.5%) (Table 5).

The distribution of virulence genes in the *Enterococcus* species as shown in table 6 revealed the presence of *gel*E 120 (85.1%), *cyl*A 52 (36.9%), *hyl* 96 (68.1%), *esp* 135 (95.8%), *ace* 127 (90.1%) and *agg* 118 (83.7%). All enterococci isolates (with the exemption of five strains of *Enterococcus hirae*) that genetically harboured the *gel*E phenotype produced gelatinase activity (Compare figure 1 and table 6). Likewise, all enterococci isolate (with the exemption of five strains of other *Enterococcus* species) that harboured the *cyl*A gene, phenotype expressed beta-lactamase activity (Compare figure 1 and table 6). Furthermore, all enterococci isolate with the *esp* gene expressed S-layer production phenotypically (Compare figure 1 and table 6). A significantly positive correlation exists between the beta-haemolytic activity of the *Enterococcus* species and *cyl*A gene detected in this study ($p<0.05$). Likewise, a significant positive correlation was observed in this study between S-layer, *ace*, *esp* and *gel*E genes ($p<0.05$).

Table 5: Distribution of Antibiotic Resistant Genes in the *Enterococcus* species

Target genes	*E. faecalis* *n*=36		*E. faecium* *n*=39		*E. durans* *n*=19		*E. casseliflavus* *n*=13		*E. hirae* *n*=14		Other *Enterococcus* species *n*=20		Total *n*=141	
	PR	GR	PR	GR	PR	GR	PR	GR	PR	GR	PR	GR	PR	GR
*van*A		17 (73.9)		21 (80.8)		4 (66.7)		5 (100)		4 (66.7)		4 (50)		55 (74.3)
*van*B	23	13 (56.5)	26	18 (69.2)	6	5 (83.3)	5	5 (100)	6	2 (33.3)	8	7 (87.5)	74	50 (67.6)
*van*C21/2		1 (4.4)		2 (7.7)		0 (0)		0 (0)		0 (0)		1 (12.5)		4 (5.4)
*tet*A		17 (80.9)		16 (88.9)		7 (70)		2 (50)		3 (60)		5 (55.6)		50 (74.6)
*tet*B	21	0 (0)	18	0 (0)	10	0 (0)	4	0 (0)	5	0 (0)	9	0 (0)	67	0 (0)
*tet*C		20 (95.2)		18 (100)		9 (90)		3 (75)		4 (80)		7 (77.8)		61 (91.1)
bla_{TEM}	36	34 (94.4)	39	37 (94.9)	19	11 (57.9)	13	7 (53.9)	14	8 (57.1)	20	13 (65)	141	110 (78)
*bla*pse1		36 (100)		39 (100)		9 (47.4)		10 (76.9)		11 (78.6)		17 (85)		122 (86.5)
*erm*A		22 (75.9)		26 (81.3)		5 (83.3)		3 (30)		2 (40)		4 (44.4)		62 (68.1)
*erm*B	29	17 (58.6)	32	21 (65.6)	6	2 (33.3)	10	6 (60)	5	3 (60)	9	6 (66.7)	91	55 (60.4)
*erm*C		2 (6.9)		3 (9.4)		0 (0)		0 (0)		0 (0)		0 (0)		5 (5.5)

Legend: PR: Phenotypic resistance; GR: Genotypic resistance; values in parenthesis represent percentage (%)

Table 6: Distribution of Virulence Genes in the *Enterococcus* species

Target genes	*E. faecalis* *n*=36	*E. faecium* *n*=39	*E. durans* *n*=19	*E. casseliflavus* *n*=13	*E. hirae* *n*=14	Other *Enterococcus* species *n*=20	Total *n*=141
*gel*E	33 (91.7)	35 (89.7)	14 (73.7)	10 (76.9)	12 (85.7)	16 (80)	120 (85.1)
*cyl*A	12 (33.3)	15 (38.5)	4 (21.1)	7 (53.9)	5 (35.7)	9 (45)	52 (36.9)
hyl	28 (77.7)	22 (56.4)	12 (63.2)	10 (76.9)	11 (78.6)	13 (65)	96 (68.1)
esp	36 (100)	38 (97.4)	17 (89.5)	12 (92.3)	14 (100)	18 (90)	135 (95.8)
ace	32 (88.9)	36 (92.3)	15 (78.9)	13 (100)	13 (92.9)	18 (90)	127 (90.1)
agg	29 (80.6)	33 (84.6)	17 (89.5)	10 (76.9)	12 (85.7)	17 (85)	118 (83.7)

Values in parenthesis represent percentage (%)

Biofilm formation capacity of the enterococci isolates as shown in table 7 demonstrated non-producers (25 (17.7%), weak producers (26 (18.4%), moderate producers (35 (24.8%) and strong producers (55 (39.0%).

Antibiotic resistant *Enterococcus* species with biofilm formation potential as shown in table 8 revealed penicillin + biofilm (116 (82.3%), chloramphenicol + biofilm (116 (82.3%), clindamycin + biofilm (108 (76.6%), erythromycin + biofilm (85 (60.3%), streptomycin + biofilm (99 (70.2%), vancomycin + biofilm (74 (52.3%), imipenem + biofilm (55 (39.0%), meropenem + biofilm (42 (29.8%), ciprofloxacin + biofilm (34 (24.1%), kanamycin + biofilm (40 (28.4%) and piperacillin + biofilm (22 (15.6%).

Table 7: Distribution of Biofilm Formation among the *Enterococcus* species

Biofilm Indicators	*E. faecalis* *n*=**36**	*E. faec ium* *n*=**39**	*E. durans* *n*=**19**	*E. casseliflavus* *n*=**13**	*E. hirae* *n*=**14**	**Other** *Enterococcus* **species** *n*=**20**	**Total** *n*=**141**
Non producers	0 (0)	7 (17.9)	8 (42.1)	0 (0)	1 (7.1)	9 (45)	25 (17.7)
Weak producers	2 (5.5)	5 (12.8)	7 (36.8)	4 (30.8)	5 (35.7)	3 (15)	26 (18.4)
Moderate producers	8 (22.2)	13 (33.3)	3 (15.8)	3 (23.1)	5 (35.7)	3 (15)	35 (24.8)
Strong producers	26 (72.2)	14 (35.9)	1 (5.3)	6 (46.2)	3 (21.4)	5 (25)	55 (39.0)

Values in parenthesis represent percentage (%)

Table 8: Antibiotic Resistant *Enterococcus* species with Biofilm Formation Potential

Antibiotics	**Resistant strains** *n*=**141 (%)**	**Resistant strains with biofilm formation potential** *n*=**141 (%)**				**Total biofilm producers with antimicrobial potential** *n*=**141 (%)**
		Non producers *n*=**25 (%)**	**Weak producers** *n*=**26 (%)**	**Moderate producers** *n*=**35 (%)**	**Strong producers** *n*=**55 (%)**	
VAN	74 (52)	0 (0)	2 (7.6)	17 (48.6)	55 (100)	74 (52.3)
TET	67 (47)	5 (20)	4 (15.4)	15 (42.9)	43 (78.2)	62 (43.9)
CIP	34 (24)	0 (0)	4 (15.4)	3 (8.6)	27 (49.1)	34 (24.1)
MEM	42 (29)	0 (0)	2 (7.6)	8 (22.9)	32 (58.2)	42 (29.8)
IMI	59 (41)	4 (16)	6 (23.1)	10 (28.6)	39 (70.9)	55 (39.0)
CLI	131 (93)	23 (92)	18 (69.2)	35 (100)	55 (100)	108 (76.6)
KAN	42 (29)	2 (8)	0 (0)	2 (5.7)	37 (67.3)	40 (28.4)
STR	104 (73)	5 (20)	9 (34.6)	35 (100)	55 (100)	99 (70.2)
ERY	91 (64)	6 (24)	3 (11.5)	30 (85.7)	52 (94.5)	85 (60.3)
CHL	141 (100)	25 (100)	26 (100)	35 (100)	55 (100)	116 (82.3)
PIP	29 (20)	7 (28)	2 (7.6)	5 (14.3)	15 (27.3)	22 (15.6)
PEN	141 (100)	25 (100)	26 (100)	35 (100)	55 (100)	116 (82.3)

Legend: VAN: Vancomycin (30 µg); TET: Tetracycline (30 µg); CIP: Ciprofloxacin (50 µg); MEM: Meropenem (10 µg); IMI: Imipenem (10 µg), CLI: Clindamycin (2 µg), KAN: Kanamycin (30 µg); STR: Streptomycin (10 µg); ERY: Erythromycin (15 µg); CHL: Chloramphenicol (30 µg), PEN: Penicillin G (10 µg), and PIP: Piperacillin (100 µg). Values in parenthesis represent percentage (%)

DISCUSSION

The capacity to acquire resistance to several classes of antimicrobials constitutes a significant feature of *Enterococcus* species. This does not only indicate serious therapeutic implication but also creates an environmental reservoir of antimicrobial resistance and virulence genes, often associated with mobile genetic elements, such as plasmids and conjugative transposons (Igbinosa and Beshiru, 2019). In the present study, we characterized the biofilm formation, antimicrobial resistance and virulence of *Enterococcus* species

recovered from aquaculture and slaughterhouse environments. The distribution of *Enterococcus* species in this study revealed *Enterococcus faecalis (E. faecalis)*, *E. faecium*, *E. durans*, *E. casseliflavus*, *E. hirae* and other *Enterococcus* species.

Studies from previous authors have also reported similar *Enterococcus* species from environmental sources (Sadowy and Luczkiewicz, 2014). More than 50% of the enterococci isolates by Ndubuisi *et al.* (2017) were resistant to doxycycline and erythromycin which is in agreement with the findings in this study. In addition, antimicrobial susceptibility testing by Sadowy and Luczkiewicz (2014) revealed the resistance to tetracycline, erythromycin, aminoglycosides, ampicillin and fluoroquinolones, similar to the multidrug resistance profile of isolates in this study. All isolates in this study were resistant to penicillin G. In addition, 78% and 86.5% of the isolates in this study harboured the bla_{TEM} and *bla*pse1 genes respectively.

High-level resistance to â-lactam in *Enterococcus* species is principally attributed to two mechanisms: an assembly of the enzyme â-lactamases or the assembly of low-affinity penicillin-binding protein 5 (PBP5) (Arias *et al.*, 2010). Continuous production of PBP5 with reduced-affinity covalently binding to â-lactams is attributed to *E. faecium* but infrequent among *E. faecalis*. Beta-lactamases production is rare in enterococci and can result in high-level resistance via cleavage of â-lactams prior to their target site (cell wall).

Glycopeptides (teicoplanin and vancomycin), are active agents of the cell wall with antibacterial effect by covalently binding with elevated affinity to D-Ala-D-Ala terminal of the precursors of pentapeptide to prevent the peptidoglycan synthesis (Walsh, 2000). Eight variations of acquired glycopeptide resistance (*van*N, *van*M, *van*L, *van*G, *van*E, *van*D, *van*B, and *van*A) in enterococci have been described (O'Driscoll and Crank, 2015). The *van*A gene is responsible for most human vancomycin-resistant enterococci (VRE) cases globally, mostly expressed by *E. faecium* (O'Driscoll and Crank, 2015). Bhardwaj *et al.* (2016) reported that chlorhexidine induces the expression of *van*A-type vancomycin resistance genes as well as genes connected with the resistance of daptomycin. Multiple antibiotic-resistant enterococci in the population represent a threat to human safety. Enterococci easily acquire resistance when exposed to antibiotics or when they acquire genetic resistance factors from neighbouring organisms (Beshiru *et al.*, 2017).

In a study by Heidari *et al.* (2017), *asa*1 gene was the most frequently detected gene (100%) among the isolates, followed by *gel*E (80.4%), *cyl*A (64.7%), and *byl* (51%) which was higher than the virulence determinants detected in this study. Extracellular surface protein (*esp*) is a virulence factor that contributes to biofilm formation and resistance to environmental stresses (Chajecka-Wierzchowska *et al.*, 2017; Igbinosa and Beshiru, 2019). It encourages colonization, adhesion and evasion of the immune system with a crucial role in antibiotic resistance. Kafil and Mobarez (2015) reported a significant correlation between *esp* harbouring *E. faecium* and vancomycin resistance; while *E. faecalis* was reported to correlate with *esp* harbouring enterococci and resistance to tetracycline, chloramphenicol and ampicillin respectively. Studies on the genetics of acquisition and dissemination of resistance and virulence genes, suggests that genetic resistance and virulence can spread through the population via human, environmental or animal reservoirs (Deshpande *et al.*, 2015).

Resistance to erythromycin in *Enterococcus* species is by methylation of the 23S rRNA enzymes methylase encrypted by *erm* genes and other elements involved the macrolides, lincosamides and streptogramins (MLS) bacterial resistance (Giguère, 2013). MLS bacterial resistance is encrypted by the prevalent *erm*B determinant and occasionally through *erm*C or *erm*A. Erythromycin resistance methylases (*erm*) in bacteria express resistance through modification of A2058 nucleotide of 23S rRNA (methylation), leading to resistance in MLS antibiotics. The *erm*B genetic element is distributed among enterococci, particularly *E. faecium* and *E. faecalis* (Jensen *et al.*, 1999). The *erm*B genetic element form part of multi-resistance plasmids often linked with Tn1546-like *van*A elements (Laverde-Gomez *et al.*, 2010).

In strains of *E. faecalis*, substantive virulence determinants have been elucidated which may adhere to host cells and colonize the mucosal surfaces resulting in infection (Aslam *et al.*, 2012). Such colonization and interactions are referred to as preliminary steps in triggering pathogenesis in many infectious agents. The *ace* gene (adhesion of the collagen gene) encodes a protein that mediates the connection of bacteria to protein matrix of host cells (Chajecka-Wierzchowska *et al.*, 2017). The *esp* gene (enterococcal surface protein gene) encodes a surface protein involved in pathogenesis through adhesion characteristics of enterococcal cells to abiotic and biotic surfaces and formation of biofilm. The gelatinase (*gel*E) is a metalloproteinase found in endocarditis and contributes to virulence (Aslam *et al.*, 2012).

A considerable prevalence of genotypic virulence elements (*gel*E, *esp* and *ace*) was observed in a good number of the isolates in this study; in agreement with findings from previous studies (Aslam *et al.*, 2012). A significantly positive correlation exists between the beta-haemolytic activity of the *Enterococcus* species and *cyl*A gene detected in this study ($p<0.05$). The surface layer (S-layer) form an integral part of the cell envelope that exists in most bacteria. It is made up of a monomolecular coating composed of identical glycoproteins or proteins (Igbinosa and Beshiru, 2019). In many species of bacteria, the S-layer is the sole component of the cell wall and, thus important for osmotic and mechanical stabilization (Igbinosa and Beshiru, 2019).

A significant positive correlation was observed in this study between S-layer, *ace*, *esp* and *gel*E genes ($p<0.05$). Enterococci can infect humans and food animals due to their virulence determinants associated with the formation of biofilm including aggregation substance, gelatinase, enterococcal surface protein and capsule formation (Chajecka-Wierzchowska *et al.*, 2017). *E. faecalis* has been described by Chajecka-Wierzchowska and co-worker as the most common cause of 80-90% of infection followed by *E. faecium* with 10-15% of infection (Chajecka-Wierzchowska *et al.*, 2017). The emergence of enterococci with multidrug resistance coupled with the formation of biofilm to vancomycin is predominantly seen in *E. faecium* (Borhani *et al.*, 2014). Many strains have been reported to be resistant to one or more antibiotics, including vancomycin (£uczkiewicz *et al.*, 2010). Sadowy and Luczkiewicz (2014) reported that their isolates not only carried several resistance determinants but were also enriched in genes encoding pathogenicity adherent factor (*esp*) which is in line with the findings in this study. To strategically minimize the threat to human health from antibiotic resistance, understanding its drivers and mechanisms is essential.

CONCLUSION

The present study has revealed the diversity of *Enterococcus* species from slaughterhouse and aquaculture effluents in Benin City, Nigeria. In addition, the genetic variability of the enterococcal isolates was characterized. Findings from this study revealed that pathogenic enterococci which inhabit non-human reservoirs could play a key role in the spread and acquisition of antibiotic resistance elements. Since humans play a significant role in the aquaculture and slaughterhouse environments to meet the food demand of the ever-increasing populations, it is necessary to control the discharge of untreated effluents from aquaculture and slaughterhouse to the surrounding environments so as to control the dissemination of antibiotic-resistant bacteria clones in the environment. There is a need for environmental risks communiqué to stakeholders on proper environmental management and the administration of antimicrobial agents and or/ biocides.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

REFERENCES

Arias, C.A., Contreras, G.A. and Murray, B.E. (2010) Management of multidrug-resistant enterococcal infections. *Clinical Microbiology and Infection,* 16(6): 555-562.

Aslam, M., Diarra, M.S., Checkley, S., Bohaychuk, V. and Masson, L. (2012) Characterization of antimicrobial resistance and virulence genes in *Enterococcus* spp. isolated from retail meats in Alberta, Canada. *International Journal of Food Microbiology,* 156: 222-230.

Beshiru, A., Igbinosa, I.H., Omeje, F.I., Ogofure,

A.G., Eyong, M.M. and Igbinosa, E.O. (2017) Multi-antibiotic resistant and putative virulence gene signatures in *Enterococcus* species isolated from pig farms environment. *Microbial Pathogenesis,* 104: 90-96.

Beshiru, A. and Igbinosa, E.O. (2018) Characterization of extracellular virulence properties and biofilm-formation capacity of *Vibrio* species recovered from ready-to-eat (RTE) shrimps. *Microbial Pathogenesis* 119: 93-102.

Bhardwaj, P., Ziegler, E., Adams, H. and Palmer, K.L. (2016) Chlorhexidine induces *van*A-type vancomycin resistance genes in enterococci. *Antimicrobial Agents and Chemotherapy,* 60: 2209-2221.

Borhani, K., Ahmadi, A., Rahimi, F., Pourshafi, M.R. and Talebi, M. (2014) Determination of vancomycin-resistant *Enterococcus faecium* diversity in Tehran sewage using plasmid profile, biochemical fingerprinting and antibiotic resistance. *Jundishapur Journal of Microbiology,* 7(2): 8951-8958.

Cattoir, V. and Leclercq, R. (2013) Twenty-five years of shared life with vancomycin-resistant enterococci: is it time to divorce? *Journal of Antimicrobial Chemotherapy,* 68(4): 731-742.

Chajecka-Wierzchowska, W., Zadernowska, A. and Łaniewska-Trokenheim, L. (2016) Diversity of antibiotic resistance genes in *Enterococcus* strains isolated from ready-to-eat meat products. *Journal of Food Science,* 81: M2799-807.

Chajecka-Wierzchowska, W., Zadernowska, A. and Łaniewska-Trokenheim, L. (2017) Virulence factors of *Enterococcus* spp. presented in food. *LWT - Food Science and Technology,* 75: 670-676.

Clinical and Laboratory Standards Institute (CLSI) (2017) Performance Standards for Antimicrobial Susceptibility Testing M02-A12, M07-A10, and M11-A8. 27th Edition; 282pp.

Daniel, D.S., Lee, S.M., Dykes, G.A. and Rahman, S. (2015) Public health risks of multiple-drug-resistant *Enterococcus* spp. in Southeast Asia. *Applied and Environmental Microbiology,* 81: 6090 -6097.

Deshpande, L.M., Ashcraft, D.S., Kahn, H.P., Pankey, G., Jones, R.N., Farrell, D.J. and Mendes, R.E. (2015) Detection of a new *cfr*-like gene, *cfr*(B), in *Enterococcus faecium* isolates recovered from human specimens in the United States as part of the SENTRY antimicrobial surveillance program. *Antimicrobial Agents and Chemotherapy,* 59: 6256-6261.

Elhani, D., Klibi, N. and Dziri, R. (2014) *van*A-containing *E. faecium* isolates of clonal complex CC17 in clinical and environmental samples in a Tunisian hospital. *Diagnostic Microbiology and Infectious Disease,* 79: 60-63.

Food and Agriculture Organization (FAO) of the United Nations (2012) Proceedings of the International Workshop on the Use of Antimicrobials in Livestock Production and Antimicrobial Resistance in the Asia-Pacific Region 2012. Food and Agriculture Organization of the United Nations, Rome, Italy.

Frieri, M., Kumar, K. and Boutin, A. (2017) Antibiotic resistance. *Journal of Infection and Public Health,* 10: 369-378.

Giguère, S. (2103) Lincosamides, Pleuromutilins, and Streptogramins, In Giguère, S., Prescott, J.F., Dowling, P.M. (Eds.) Antimicrobial Therapy in Veterinary Medicine. John Wiley & Sons, Inc, Oxford, UK, 199-231.

Heidari, H., Hasanpour, S., Ebrahim-Saraie, H.S. and Motamedifar, M. (2017) High Incidence of virulence factors among clinical *Enterococcus faecalis* isolates in South-Western Iran. *Journal of Infection and Chemotherapy,* 49(1): 51-56.

Igbinosa, E.O. and Beshiru, A. (2019) Antimicrobial resistance, virulence determinants, and biofilm formation of *Enterococcus* species from ready-to-eat seafood. *Frontiers in Microbiology,* 10: 728.

Jensen, L.B., Frimodt-Moller, N. and Aarestrup, F.M. (1999) Presence of *erm* gene classes in Gram-positive bacteria of animal and human origin in Denmark. *FEMS Microbiology Letters,* 170: 151-158.

Kafil, H.S. and Mobarez, A.M. (2015) Spread of enterococcal surface protein in antibiotic resistant *Enterococcus faecium* and

Enterococcus faecalis isolates from urinary tract infections. *Open Microbiology Journal,* 9: 14–17.

Kang, C.I. and Song, J.H. (2013) Antimicrobial resistance in Asia: Current epidemiology and clinical implications. *Infection and Chemotherapy,* 45: 22–31.

Laverde-Gomez, J.A., Van, S.W., Freitas, A.R., Coque, T.M., Weaver, K.E., Francia, M.V., Witte, W. and Werner, G.A. (2010) A multi-resistance mega-plasmid pLG1 bearing a *hyl*(Efm) genomic island in hospital *Enterococcus faecium* isolates. *International Journal of Medical Microbiology,* 23: 345-352.

£uczkiewicz, A., Jankowska, K., Fudala-Ksiazek, S. and Olanczuk-Neyman, K. (2010) Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Research,* 44: 5089-5097.

Marshall, B.M. and Levy, S.B. (2011) Food animals and antimicrobials: Impacts on human health. *Clinical Microbiology Reviews,* 24: 718-733.

Ndubuisi, J.C., Olonitola, O.S., Olayinka, A.T., Jatau, E.D. and Iregbu, K.C. (2017) Prevalence and antibiotics susceptibility profile of *Enterococcus* spp. isolated from some Hospitals in Abuja, Nigeria. *African Journal of Clinical and Experimental Microbiology,* 18(3): 154-158.

O'Driscoll, T. and Crank, C.W. (2015) Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. *Infection Drug Resistance,* 8: 217-230.

Rathnayake, I.U., Hargreaves, M. and Huygens, F. (2012) Antibiotic resistance and virulence traits in clinical and environmental *Enterococcus faecalis* and *Enterococcus faecium* isolates. *Systematic and Applied Microbiology,* 35: 326-333.

Sadowy, E. and Luczkiewicz, A. (2014) Drug-resistant and hospital-associated *Enterococcus faecium* from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. *BMC Microbiology,* 14: 66-681.

Staley, C., Dunny, G.M. and Sadowsky, M.J. (2014) Environmental and animal-associated enterococci. *Advances in Applied Microbiology,* 87: 147-186.

von Wintersdorff, C.J., Penders, J., van Niekerk, J.M., Mills, N.D., Majumder, S. and van Alphen, L.B. (2016) Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Frontiers in Microbiology,* 7: 173.

Walsh, C. (2000) Molecular mechanisms that confer antibacterial drug resistance. *Nature*, 406: 775-781.