Alfarama Journal of Basic & Applied Sciences

Faculty of Science Port Said University

https://ajbas.journals.ekb.eg
ajbas@sci.psu.edu.eg
http://sci.psu.edu.eg/en/

April 2023, Volume 4 , Issue II

DOI:https://doi.org/10.21608/ajbas.2022.162053.1121

ISSN 2682-275X

Submitted: 10/09/2022
Accepted: 04/12/2022

Pages: 290 - 309

Prevalence, Species Composition, Antibiogram and Vancomycin Resistant Determinant of *Enterococcus* spp. Isolated from Wastewater Treatment Plant and its Affected Ecosystems

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ABSTRACT

Most local and international legislation neglect the risk of transferring pathogenic bacteria, especially those with multiple antibiotic resistance (MAR), and causal genes. Egypt lacks data on the prevalence of MDR/VRE enterococci in wastewater reclaimed soils or aquatic ecosystems receiving treated wastewater. Therefore, this study goals are to study the prevalence of MDR/VRE enterococci in wastewater treatment system, reclaimed soil and effluent receiving water bodies. Monthly schemes were implemented from February 2018 to January 2019 to collect samples of raw and treated sewage, sludge, reclaimed soil, drainage canal, and downstream lake. Samples for total enterococci were analyzed using the Multiple Tube method. Enterococci isolates were purified and confirmed to species level by PCR. Antibiotics sensitivity testing was performed by Disk Diffusion method. Ampicillin, chloramphenicol, ciprofloxacin, gentamicin, erythromycin, tetracycline, and vancomycin were tested for antibiotic susceptibility using the Kirby bauer method. In addition, *van*A and *van*B genes were screened in the confirmed VRE strains. Although, the wastewater treatment plant was able to eliminate 98% of total enterococci, the discharged or reclaimed effluent still containing 3 LOG MPN/ 100 mL of enterococci, mostly (64%) MDR *Enterococcus faecalis* and *Enterococcus faecium* with MARI >0.2. VRE were detected in 7% of samples, including reclaimed water and receiving water bodies regimen was not effective in removing VRE and multi-antibiotic-resistant enterococci (MAR). These findings demonstrate that MDR/VRE enterococci are released into the environment via wastewater, where they potentially pose a concern to human health.

Key Words:

Enterococcus spp., Antibiogram, Reclaimed soil, VRE, Wastewater treatment.

1. INTRODUCTION

Enterococci belong to lactic acid-producing bacteria, which are Gram-positive, catalase-negative catalase and non-spore producers, and many of them are characterized by the production of anti-bacterial peptides bacteriocins [1], [2], [3]. Enterococci coexist within the normal flora in the humans and animals' gastrointestinal tract without causing infection [4], [5]. Due to their occurrence in human and animal gut, enterococci, especially faecal-origin enterococci such as *E. faecalis, E. faecium, E. hirae, and E. durans*, are regarded as general indicator of faecal contamination or pathogen contamination in risk assessment and other monitoring programs [6]–[9].

Enterococci are opportunistic pathogens that cause millions of infections annually. Although strains of *E. faecalis, E. faecium, E. hirae, and E. durans* are considered harmless to healthy people, they can be pathogenic for those with immunodeficiency or severe disease [10]. The genome of these strains gains and disseminate resistance to the most commonly used antibiotics, including those used to treat severe infections, making them difficult to treat [11], [12]. Numerous studies have warned about *Enterococcus* spp. presence in wastewater treatment plants, stressing that these types of drug-resistant bacteria pose a global threat to public health; because it causes serious illness, and sometimes death [13], [14].

The ability of bacteria in the genus *Enterococcus* to acquire and spread determinants of resistance to antimicrobials is a major characteristic of these organisms. Resistance to chloramphenicol, erythromycin, tetracycline, fluoroquinolones, and vancomycin is an example of acquired resistance, while resistance to penicillin, cephalosporins, amino glycosides, and clindamycin is due to the various intrinsic traits they express [15]–[17]. For nearly 30 years after its medical introduction, vancomycin-resistant enterococci (VRE) have been reported, and the trend has only worsened since that time [18]. Early in the 1990s, doctors began discovering vancomycin-resistant *E. faecalis* and *E. faecium* strains among clinical isolates [19]. Since its identification, vancomycin-resistant enterococci (VRE) have garnered special attention from a standpoint of healthcare and are recognized in the World Health Organization as a priority emerging pathogen within ESKAPE pathogens [20]–[23]. Endocarditis, UTIs, prostatitis, cellulitis, wound infections, and concomitant bacteremia have all been linked to *Enterococcus* spp., especially *E. faecium* and *E. faecalis* [13], [15], [24], [25]. Similarly, vancomycin-resistant enterococcal strains are increasingly prevalent both in and outside of healthcare facilities, and this resistance is frequently accompanied by the presence of multiple antibiotic-resistant strains, which poses a serious threat to public health given the depletion of treatment options [19], [26], [27]. The fact that bacteria resistant to vancomycin can pass on the resistance genes to other bacteria is dangerous to human health. *Van*A, B, C, D, and E are just some of the many vancomycin resistance phenotypes that have been identified [20], [28], [29]. Most studies on resistance phenotypes *van*A and *van*B have focused on *E. faecalis* and *E. faecium* [20], [28], [30], [31]. *Van*C resistance phenotypes have been described in *E. coli*, *Salmonella*, and Enterobacteriacea [32].

The purpose of this investigation was to characterize the antimicrobial resistance profiles, antimicrobial determinants of *Enterococcus* spp. recovered from municipal wastewaters in Sarabium, Ismailia, Egypt and its affected environmental reservoirs and reclaimed soil.

2. STUDY AREA

Site Description: Wastewater samples were collected from Sarabium wastewater treatment plant (Figure 1, 1-a) in Ismailia, Egypt. The wastewater treatment plant at Sarabium was inaugurated in 1996 on an area of 860 acres with a design capacity of 270,000 m^3/day and an average operating capacity of 170,000-220,000 m^3/day. The plant employs a standard secondary biological treatment for wastewater. The station serves 650 000 people in Ismailia, Abu Sultan, Sarabium, Mostakbal City, Nafisha, and Bahtimi, and receives wastewater from multiple hospitals and a pharmaceutical

plant. The final effluent is discharged from the plant in two main directions: to the artificial forest area, where 50,000 m^3/day of treated water is passed through a pre-filtration system for irrigation and used to irrigate the experimental forest lands adjacent to the plant and planted with species of trees that produce timber of economic return; the remaining quantities of treated water (about 155,000 m^3/day) are discharged through a slope line of up to 11 km in length that ends at Al-Mahsama drain at 4.5 km near the Ismailia-Suez agricultural road (Figure 1-b).

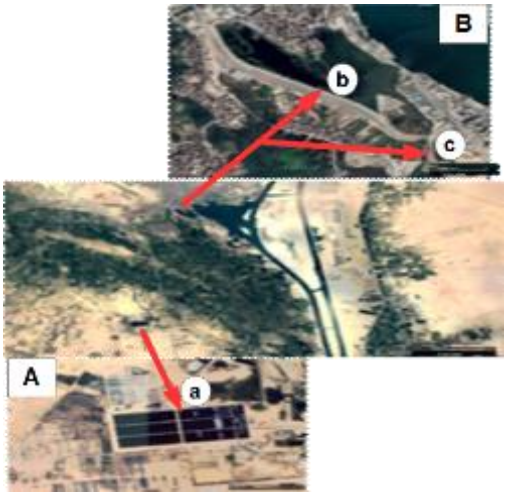

Fig. 1. Google map showing (A) Sarabium wastewater treatment facility and the adjacent reclaimed forest (a) and (B) El Sayadeen lake (b) and ElHalous drain (c), respectively.

3. MATERIALS AND METHODS

3.1 Water, soil and Wastewater Sample Collection, Transport, Preservation, and Storage: A 500 ml volume of water and wastewater from influent, effluent, El Mahsamah Drainage, and Lake El Sayadeen were collected in pre-sterilized bottles for microbiological examination. Soil and sludge samples were collected in sterile sealed plastic bags. From February 2018 to January 2019, monthly samples were collected, transported in ice box and maintained at 4°C until processing within 6 hours [33].

3.2 Presumptive Enumeration of Enterococci/Streptococci by Multiple-Tube Technique: Presumptive faecal enterococci/streptococci were counted monthly using standard technique, 9230-B [33]. Briefly, 10 mL sample/or sample dilutions were inoculated into 10 mL of 2X Azide Dextrose broth (HiMedia, India). After 24 – 48 hours at 35°C, growth (turbidity) was checked. MPN was assessed using protocol 9221-C [33]. Ten grams of sludge or soil was suspended in 100 mL PBS and shaken for 15 minutes before being diluted and processed according to protocol 9221-C [33]. For *Enterococcus* spp. confirmation, Azide Dextrose-positive tubes were streaked over Bile Esculin Azide agar (HiMedia, India). Brown halos in brownish-black colonies suggest the presence of faecal streptococci and *Enterococcus* sp. Growth in BHI broth with 6.5% NaCl (35°C for 24 h) and BHI agar at 10°C for 48 h confirmed enterococci [33].

3.3 *Enterococcus* spp. strains isolation: Ten grams of soil and sludge samples were suspended in 100 mL PBS and shaked for 15 min, let standing for 10 min and filtered through Whatman filter paper No. 1 followed by No. 20. Water and wastewater samples were processed similarly to remove bulk solids. Ten milliliters of the pre-filtered samples with appropriate dilution (to give 30-300 colonies) were filtered through 0.45 nitrocellulose filter and placed onto M-Enterococcus agar (HiMedia, India) plates

supplemented with 0.5 ml/ L tween 80 and 2 ml of 10% Sodium Carbonate solution, incubated at 35°C for 48 h. *Enterococcus* spp. colonies were selected randomly as Pink to dark red colonies [34].

3.4 DNA isolation, PCR confirmation and *Enterococcus* Species composition: For DNA isolation, a loopful of bacterial colony was suspended in 500 µL sterile MilliQ water (Merck, Germany) in an Eppendorf's tube, boiled at 95 – 100°C for 10 min, centrifuged at 15000 rpm for 10 min, and stored at -20°C until use [35]. *Enterococcus* spp. confirmation was accomplished through PCR detection genus specific *tuf* gene using primers Ent15′-TACTGACAAACCATTCATGATG-3′ and Ent2 5′-AACTTCGTCACCAACGCGAAC-3′ [36]. PCR was performed in 20 uL volume using 2x PCR Master mix (Promega, USA). thermal cycle running conditions was one cycle for 3 min at 95°C, 35 cycles of 30 s at 55°C, and 1 min at 72°C, with final extension at 72°C for 7-min was used, the expected product size is 112 bp [36]. Polymerase Chain Reaction was used to determine the species of *Enterococcus*, as described by Jackson [37]. Primers, fragment length and annealing temperature are shown in table 1. The reaction mixture was conducted in 20 ul reaction volume using 2X Master Mix (Promega, Germany) in compliance with the instruction manual. PCR fragments were visualized by electrophoresis in 1.5% gel by UV transilluminator (Maestrogen, Taiwan) using Ethidium Bromide [38], 100-bp and 1Kbp ladders were used as DNA molecular weight marker. *E. faecium* and *E. coli* were used as positive and negative controls, respectively

Table 1. PCR primers used for *Enterococcus* species differentiation.

Strain	Primer	Sequence (5′–3′)	Product Size(bp)	Annealing	Ref.
E.faecalis	FL1 FL2	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	360	48	[37]
E.durans	DU1 DU2	CCTACTGATATTAAGACAGCG TAATCCTAAGATAGGTGTTTG	295	52	[37]
E.casseliflavus	CA1 CA2	TCCTGAATTAGGTGAAAAAAC GCTAGTTTACCGTCTTTAACG	288	52	[37]
E.faecium	FM1 FM2	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATTCTTCTTTA	215	52	[37]
E.hirae	HI1 HI2	CTTTCTGATATGGATGCTGTC TAAATTCTTCCTTAAATGTTG	187	48	[37]

3.5 Antimicrobial Susceptibility Testing: Disk diffusion method was used to determine antimicrobial susceptibilities of *Enterococcus* strains [39] to Tetracycline (30 µg), Vancomycin (30 µg), Norfloxacin (10 µg), Ciprofloxacin (5 µg), Azithromycin (15 µg), Erythromycin (15 µg), Ampicillin (10 µg), and Chloramphenicol (30 µg) (oxoid, England) on Muller Hinton agar (Oxoid, England). *E. coli* ATCC 25922 employed for quality-control for antibiotic tests. The Multiple Antibiotic Resistance index (MARI) for each enterococci isolate in this study was calculated in accordance with Equation introduced by Krumperman, 1983 [40]. MARI= a/b where a is the number of antibiotics to which strain is resistant, and b is the total number of antibiotics tested in the experiment.

3.6 Statistical analysis: Statistics analysis including descriptive analysis and one-way analysis of variance was performed using SPSS Statistics software V. 23.0 (IBM SPSS Statistics for Windows, NY, USA). Heatmap was created by complete linkage protocol by http://www.heatmapper.ca.

4. RESULTS AND DISCUSSION

4.1 *Enterococcus* spp. monthly prevalence at different study sites: The monthly changes in *Enterococcus* spp. (MPN /100 ml) at the sampling locations are determined and presented in Table (2). Variations in *Enterococcus* spp. was assessed using repeated measures ANOVA at 0.05 level. The average *Enterococcus* spp. throughout the timepoints in influent was 5.37E+06 MPN /100 mL, where it ranged between a minimum of 1.20E+06 MPN /100 mL to a maximum of 8.20E+06 MPN /100 mL. Three log reduction in *Enterococcus* spp. was observed in the effluent with an average count of MPN 4.53E+03 MPN /100 mL. However, the filters, soil, ElHalous drainage and Lake ElSayadeen, an average *Enterococcus* spp. count of 7.35E+02, 6.37E+02, 3.78E+03 and 4.42E+02 MPN /100 mL was measured, respectively and higher average was noticed in the sludge count (8.37E+06 MPN /100 ml). The overall variations in *Enterococcus* spp. between sludge, the filters, soil, ElHalous drain, and Lake ElSayadeen samples were assessed by paired samples t-test at 0.05 level. A highly significant difference between both influent and effluent sampling sites was revealed. Multivariate analysis of variance was also applied to assess the differences in *Enterococcus* spp. induced by time (months) and sites (sludge, the filters, soil, ElHalous drainage and Lake ElSayadeen) and interaction between previous factors. Accordingly, there was a highly significantly difference in *Enterococcus* spp. between different sites ($p<0.001$), on the other hand, significant different in timepoints (months) and interaction between months and sampling site was observed ($p<0.001$).

In the current study, approximately 98% of the *Enterococcus* abundance was eliminated, from an average of 6 log MPN /100 mL in the influent to 3 log MPN / 100mL after treatment. Consistent to our results, a removal efficiency of 97% of enterococci from a European conventional wastewater treatment plant was documented, reducing the enterococci population by 3 log MPN/100 mL starting from 6 log in the plant influent. Results showed that the microbiological quality of recipient water was compromised by the treatment and disposal effluent, with the population of *Enterococcus* decreasing by 0.83 log MPN/100 mL in reclaimed soil and drain canal upstream from the effluent discharge point and by 0.99 log MPN/100 mL in the lake downstream the drain. Faecal coliforms and Enterococci are used as indicators of human or animal origin faecal contamination in environmental samples [8], [21], [29], [34], [41]. In urban communities, water bodies serving as receptacles for municipal wastewater tend to have a greater abundance of indicator bacteria such as *Enterococcus* and coliforms [26], [42]–[44]. In addition, several studies have demonstrated that fecal contamination is associated with an increased prevalence of pathogenic microorganisms that are potentially dangerous to public health [21]–[23], [45].

4.2 *Enterococcus* spp. isolation, confirmation, and species identification: Enterococcal isolates (247) were selected from 60 samples of raw sewage water (12), sludge (12), treated wastewater (12), sludge (12) and soil reclaimed with treated sewage water (12). Samples were collected on monthly bases from February 2018 to January 2019 from Sarabium wastewater treatment system, Ismailia, Egypt. Out of 247 presumptive *Enterococcus* spp. isolates, 228 (92.31%) were confirmed as *Enterococcus* genus by PCR detection of genus-specific *tuf* gene, 42 from influent wastewater, 32 effluent, 47 sludge, 39 soil, 34 El-Mahsamah Drainages, and 34 from El-Sayadeen Lake. Representative of the molecular confirmation of *Enterococcus* isolates are shown in the gel electrophoresis image in Figure 2. The 228 confirmed enterococci were further analyzed for species identification. Discrimination to species-level was achieved molecularly through PCR using species-specific primers targeting the most abundant human-associated *enterococci* (*E. faecalis*, *E. durans*, *E. casseliflavus*, *E. faecium*, and *E. hirae*). Representative results for PCR *Enterococcus* speciation are shown in the gel electrophoresis image in Figure 3. Out of 228 *Enterococcus* sp., 116 isolates (50.88%) were confirmed as *E. faecium* (21 from influent wastewater, 18 effluent, 24 sludge, 17 forest soil, 18 El-Halous drain, and 18 from El-Sayadeen Lake), 70 (30.7%) as *E. faecalis* (14 from influent wastewater, 9 effluent, 14 sludge, 11 forest soil, 11 El-Halous drain, and 11 El-Sayadeen Lake), 17 (7.46%) as *E. hirae* (4 from influent wastewater, 2 effluent, 5 sludge, 2 forest soil, 2 El-Halous drain, and 2 El-Sayadeen Lake), 6 (2.63%) as *E. durans* (1 from El-Halous drain, and 1 El-Sayadeen Lake), 8 (3.51%) as *E. casseliflavus* (1 from influent wastewater, 1 effluent, 1 sludge, 3 forest soil, 1 El-Halous drain, and 1 El-Sayadeen Lake) while the remaining 11 (4.82%) could not be delineated by the primes used in species identification because they were outside the species that were screened (Table 3).

Several studies found that *E. faecium* and *E. faecalis* are the most prevalent enterococci in human digestive tracts [4], [22], [46], thus it stands to reason that they would also be found in high concentrations in sewage treatment plants, reclaimed land, and aquatic bodies that have been contaminated by sewage [47], [48]. The proportion of *E. faecium* or *E. faecalis* isolated from the wastewater treatment plant, effluent, reclaimed soil, and aquatic bodies represented 81.15% in this investigation. On the other hand, the prevalence of other identified enterococci (*E. durans*, *E. hirae* and *E. casseliflavus*) was lower than 10.3% in all samples. These percentages are consistent with previous research in which *E. faecalis* or *E. faecium* predominated in wastewater samples and water bodies recovered from a Tunisian sewage treatment plant [49]. In another investigation on a sewage treatment plant, where treated wastewater is used in fog irrigation, *E. faecalis* or *E. faecium* accounted for 71% of the enterococci [50]. Wastewater treatment methods as well as environmental conditions and wastewater quality are likely to influence microbial diversity [51], [52]. For example, and in contrast to this study, *E. hirae* was the highest prevalent *Enterococcus* species in wastewater in two studies conducted in the United States of America and Portugal [53], [54]. In Iran, *E. hirae* was the second most common species after *E. faecium* in hospital wastewater [55]. In the wastewater of a hospital in South Africa, it was found that *E. faecalis* was the most prevalent and *E. durans* was the second most prevalent species [34]. *E. faecium* and *E. faecalis* are the most clinically isolated enterococci from human infection [43], therefore, the prevalence of *E. faecalis* and *E. faecium* in the reclaimed water and water bodies samples examined in this study may present a potential public health risk for personnel who are highly exposed to this water sources.

Unfortunately, the chlorination unit at Sarabium WWTP is broken, therefore the effluent is not disinfected before it is released into surface water bodies, despite the fact that this practice adds to environmental contamination. Numerous research have come to the conclusion that chlorination of the effluent from WWTPs can be an efficient approach for treating enterococci in wastewater [26].

4.3 Antimicrobial Susceptibility Profile of *Enterococcus* spp.: Enterococci are intrinsically resistant to many antimicrobials including cephalosporins and can easily acquire resistance to other antimicrobials that makes them difficult to treat and can cause chronic, recurrent, and sometimes fatal infections [53]. This part aims to evaluate the antimicrobial susceptibility of *Enterococcus* spp. isolated from different wastewater treatment stages of Sarabium wastewater treatment system. Antimicrobial susceptibility of the 228 *Enterococcus* sp. to resistance to 8 antimicrobials belonging to 8 drug groups was evaluated. The phenotypic resistance patterns for the 228 isolates are shown in Table (4): Tetracycline resistance in 56.14% of the isolates, erythromycin in 53.51%, gentamicin in 36%, ciprofloxacin in 27.19%, rifampicin in 21.49%, ampicillin in 18.86%, chloramphenicol in 13.62%, and vancomycin in 9.21%.

4.4.1 Multiple Antibiotic Resistance Index (MARI) of *Enterococcus* spp.: During this study, the MARI was calculated either at the level of individual isolates or at the level of samples collected at each collecting site. Both of these approaches produced comparable results. Multiple Antibiotic index (MARI) for each enterococcal isolate in this study was calculated in accordance with Equation introduced by Krumperman, 1983 [40]. The isolate's MAR index ranged from 0 (when all antibiotics were effective) to 0.750 (when the isolate was resistant to six antibiotics). There was no isolate of enterococci that was immune to any of the antibiotics tested. As shown in table (5), there was a significant prevalence of enterococcal isolates (42.5%) resistant to three or more antibiotics in all samples collected in this study (MARI of 0.375). On the other hand, 9 (3.93%) isolates that showed resistance to five drugs (MAR index of 0.625), and 3 (1.32%) that showed resistance to six (MARI of 0.75). Additionally, there were 13 isolates that showed complete susceptibility to all antimicrobial tested, 52 (22.8%) isolates that showed resistance to only one drug (MARI of 0.125), and 66 (28.95%) isolates that showed resistance to two antibiotics (MARI of 0.25). Depending on whatever experimental environment compartment was examined, distinct behaviors were observed in the MAR index. There was an increase in the incidence of enterococci isolates (52 or 22.8%) demonstrating three or more antibiotic resistances (MARI of ≥0.375) in all samples from the wastewater treatment plant. There were 24 (57.14%) enterococci isolates with three or more antibiotic resistances and a MAR index of 0.375 or higher in influent wastewater, 22 (46.8%) in sludge, and 6 (18.75%) in effluent, respectively. Sarabium wastewater treatment plant receives wastewater from various large hospitals and companies, including a pharmaceutical industry, in in addition to domestic sewage. As a result, the input and impact of hospital and pharmaceutical waste in the prevalence of specific resistances in the wastewater treatment plant cannot be neglected, and this may explain why the largest proportion of isolates have a higher number of multiple antibiotic resistances.

Sarabium wastewater treatment plant receives wastewater from different large hospitals and different industries including pharmaceutical factory. Therefore, the input and impact of the hospitals and pharma waste in the presence of certain resistances in the wastewater treatment plant cannot be overlooked and may explain the highest percentage of isolates with a higher number of antibiotic resistances. At 57.14%, influent samples showed the highest concentration of enterococcal isolates resistant to three or more antibiotics (MARI 0.375 or higher) with 24 isolates, followed by sludge at 46.8% (n=22), and effluents at 18.75% (n=6).

After the effluent is discharged, the pattern drastically shifts, with only 15.35% (n=35) of enterococcal isolates exhibiting resistance to three or more antibiotics (MARI 0.375), and a total of 26.32% (n=60) of the isolates showing multiple antibiotic resistance of both MARI= 0.125 and 0.250. Only nine (3.95 percent) of the enterococcal isolates were fully susceptible to all tested antibiotics. The environmental samples yielded no isolates with resistance to 6 or more of the antibiotics tested; nevertheless, 9 (3.95%) and 3 (1.31%) isolates with MARI values of 0.5 and 0.625 were discovered.

For ElSayadeen lake, 29.4% (n = 10) of the isolated enterococci strains had a MARI of 0.125, whereas 26.5% (n = 9) and 23.5% (n = 8) had MARIs of 0.25 and 0.375, respectively. Although 14.6% (n=5) of the isolates were sensitive to all antibiotics, only 5.45% (n=2) of the isolates exhibited resistance to four or more drugs. Antibiotic resistance patterns were mapped out in the reclaimed soil and the ElMahsama drain. In general, the percentage of enterococci resistant to three or more antibiotics in ElMahsama drain and reclaimed soil both stayed at 16 and 10%, respectively.

According to the data found in Table 5, the MARI of *E. faecium* was greater than 0.2 in each and every one of the sampling locations. The rates in the reclaimed soil, drain, and lake were, respectively, 0.287, 0.319, and 0.236. The index that was discovered to be the highest was 0.393, which was detected in both the plant influent and the sludge. Comparatively, *E. faecalis* MARI was highest in the influent (0.384) and lowest in the reclaimed soil (0.261) and the lake (0.284). It was found that the MARI in soil and lake samples was lower than 0.2 for *E. hirae*, *E. durans*, *E. casseliflavus*, and *Enterococcus* spp.

4.4 Presence of Vancomycin-resistant enterococci (VRE) and detection of *van*A and *van*B genetic determinants: Antibiotic resistance assay showed that 9.65% (n = 21) of total enterococcal isolates were resistant to VRE. Similar to our results, VRE detection rates at four U.S. wastewater treatment plants varied from 3% to 27% [26]. The prevalence of VRE strains in one of the US WWTP was found to be significantly higher than expected (27.0%) [56]. Treatment technology, treatment stage, disinfection method, wastewater source, and type all appear to play a role in determining the prevalence of VRE in wastewater treatment plants [5], [57]. VRE strain prevalence in wastewater have been found to vary from 2% to 52% in other investigations [26], [58], [59].

In this study, prevalence rates of 2.19, 0.88% and 3.1% (n = 5, 2, and 7) of VRE were also observed in the influent, effluent, and sludge, respectively. Whereas, among all enterococci isolates included in the study, 0.44% (n = 1) from reclaimed soils, 1.75% (n = 4) from drainage, and 0.88% (n = 2) from lake were resistant to vancomycin (Figure 4).All of the VRE had a MAR score of 0.25 or higher, and the vast majority were resistant to three or more antibiotics. Our data revealed that 3.5% of *E. faecalis*, 5.3% of *E. faecium*, and 0.44% of *E. hirae* were resistant to vancomycin. The gap in the prevalence of vancomycin resistance between species becomes more obvious when comparing the overall number of isolates to the number of isolates per species (*E. faecium*, *E. faecalis* and *Enterococcus* spp.). Vancomycin-resistant *E. faecalis* isolates made up 11.43% of all verified *E. faecalis*, while VRE isolates made up just 10.34% of all *E. faecium* and 5.88 % *E. hirae* isolates.

Detection of VRE in plant effluent, which are currently being used for reclaiming woodland and discharging in drain upstream of the lake, is considered a public health alarming situation. Therefore, disinfecting the treated effluent is highly recommended and new measures for detection of antimicrobial resistant microbial load shall be considered in the wastewater discharge/reuse standards nationally and globally.

In this study, we searched for *van*A and *van*B genes in vancomycin-resistant *Enterococcus* isolates by PCR. As shown in Figure 4, the study verified the absence of these genes in 3% (n = 3) of vancomycin-resistant enterococci isolates. Of the vancomycin-resistant isolates tested, 13 tested positive for *van*A, 3 tested positive for *van*B, and 5 tested negatives for both *van*A and *van*B. As measured, *van*A predominated in reclaimed soil, while *van*A (66.7%) and *van*B (33.3%) prevailed in the isolates from Lake ElSayadeen and the ElMahsamah drain. Thus, *van*A and *van*B were the primary genetic determinants of vancomycin resistance in the current investigation.
Out of the nine identified genes (*van*A, B, C, D, E, G, L, M, and N) contributing to vancomycin resistance in enterococci [20], We revealed two genes, *van*A and *van*B, in our VRE isolates. Prior

studies have also shown that VRE concentrations decrease as wastewater treatment progresses [18], [60]. In the current study, we also found that the concentration of VRE decreased as treatment progressed at most WWTPs where samples were collected (Figure 1). The most prevalent phenotypes, *van*A and *van*B, have been described mainly in *E. faecalis* and *E. faecium* [23], [61], [62]. In South Africa, 93.3% of VRE isolated from hospital and domestic wastewater treatment plant effluents carries *Van*B, C1 and C2/3 genes [34]. *Van*A gene carrying VRE were also found in 32 (86%, n = 37) wastewater enterococcal isolates from an Italian wastewater treatment plant [56].

4.5 Correlation between antibiotic resistance and vancomycin resistance genes among *Enterococcus* spp. isolates: The correlations between vancomycin resistance gene abundance (*van*) and phenotypic resistance to antibiotics in strains isolated from different sampling sources were determined in a clustering analysis using the complete linkage and presented in a heatmap (Figure 4). Cluster analysis revealed two main clusters. The first cluster included *Enterococcus* spp. strains resistant to tetracycline and erythromycin (Figure 4-A). This agreed with previous studies that linked erythromycin and tetracycline in *Enterococcus* spp. strains obtained from sewage [63], slaughterhouse wastewater [64], and healthcare settings [65]. The second cluster (Figure 4-B) represented two subclusters; the first (figure 4-B/a) contained only gentamycin, while the second subcluster (figure 4-B/b) comprised strains harboring *van*A and *van*B genes, as well as strains resistant to vancomycin, ampicillin, ciprofloxacin, chloramphenicol, and rifampicin. The mechanism of resistance to gentamicin cannot be determined by phenotypic testing alone because enterococci have innate resistance to aminoglycosides such as gentamycin, even at low concentrations [66], [67]. This could account for the lack of an association between gentamycin resistance and any of the other tested antimicrobials in this investigation. Several studies showed the association of vancomycin resistance in *Enterococcus* spp. with v*an* A and B genes, whether in clinical samples or those isolated from sewage treatment plants or from environmental samples [68] [26], [69]. The current study showed that ampicillin resistance shares the same cluster with vancomycin resistance implying that ampicillin resistance is correlated to vancomycin resistance. According to the results of other investigations, there appears to be a relationship between vancomycin and ampicillin resistance in enterococci isolated from wastewater treatment plants [63], [67]. Other antimicrobials clustering due to possible cross-resistance or co-selection have been also suggested [70]. These phenotypes often arise due to acquisition of a single mechanism that confers resistance to multiple antimicrobials [27],[71]. The heatmap clustering showed that high number of isolates from the influent and sludge share the same cluster with strains form effluent, soil, drain and lake. Several authors highlighted the role of wastewater treatment plant in distribution of clinically important *Enterococcus* spp. into nearby environmental niches [72]–[74].

5. CONCLUSION

According to the results of this study, wastewater treatment plants are a source of multidrug-resistant enterococci. The enterococci that were found in the affected environmental and sewage samples shared resistance to several antibiotics, and there was a strong association between these two groups of isolates. detection of VRE in the wastewater and environmental samples was correlated with the ampicillin resistance phenotype. Clinically important enterococci carrying the *van*A/B genes were detectable in the effluent of the WWTP, reclaimed soil and nearby water bodies indicating insufficient removal of VRE during wastewater treatment and permanent shedding of these antibiotic resistant pathogens into the environment from this source. This represents a risk of their transmission to the environment.

The isolates also showed a correlation between resistance to vancomycin/ ampicillin and erythromycin/ tetracycline. Enterococci levels were high upstream of the wastewater release point and in reclaimed soil. Even successful WWTPs release VRE and MDR enterococci, according to these data. Hospitals should monitor enterococcus emissions, disinfect treated wastewater before disposal, and develop new wastewater treatment methods to reduce pathogen exposure.

Table 2. *Enterococcus* spp. count at different study sites and samples including Sludge, soil, El-Halous Drainage and Lake Elsayadeen

Month	*Enterococcus spp.* (Log 10 MPN/100ml)						
	Influent	Effluent	filters	Soil (Log10 MPN/g)	ElHalous Drainage	Lake ElSayadeen	Sludge (Log10 MPN/g)
Jan	6.88	3.88	2.99	2.51	3.04	2.36	6.49
Feb	6.63	3.70	2.83	2.65	2.98	2.69	5.12
Mar	6.56	3.65	2.82	2.78	3.52	2.60	6.92
Apr	6.59	3.58	2.85	2.93	3.90	2.83	6.38
May	6.16	3.77	2.91	2.91	3.58	2.63	6.57
Jun	6.68	3.79	2.96	2.95	3.44	2.61	6.43
Jul	6.49	3.54	2.95	2.93	3.58	2.70	6.68
Aug	6.08	3.02	2.81	2.76	3.73	2.84	6.52
Sep	6.65	3.81	2.70	2.67	3.57	2.37	7.73
Oct	7.29	3.65	2.83	2.83	3.57	2.51	6.96
Nov	6.39	3.39	2.91	2.87	3.81	2.78	6.90
Dec	6.91	3.55	2.75	2.59	3.38	2.51	5.80
Total (average)	6.73	3.66	2.87	2.80	3.58	2.65	6.92
ANOVA (Months)	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***
ANOVA (Sites)	<0.0001***						

***, **, ***, significant at p<0.05, 0.01, 0.001- NS, non-significant at p<0.001**

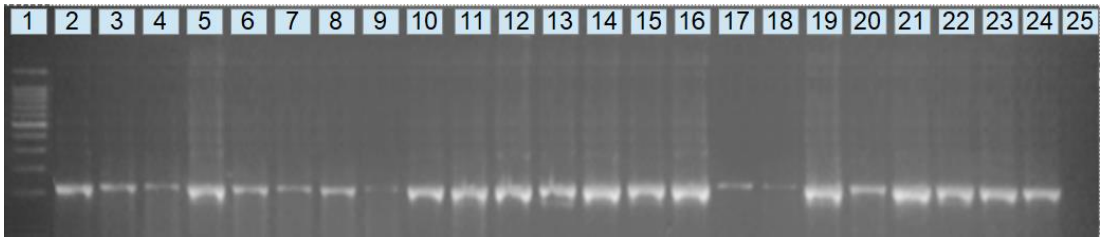

Figure (2). *Tuf* gene PCR (112 bp) for *Enterococcus* spp. confirmation. LANE 1: 1Kbp marker (Gentex), 2-23 selected isolates, 24 and 25 positive and negative controls, respectively.

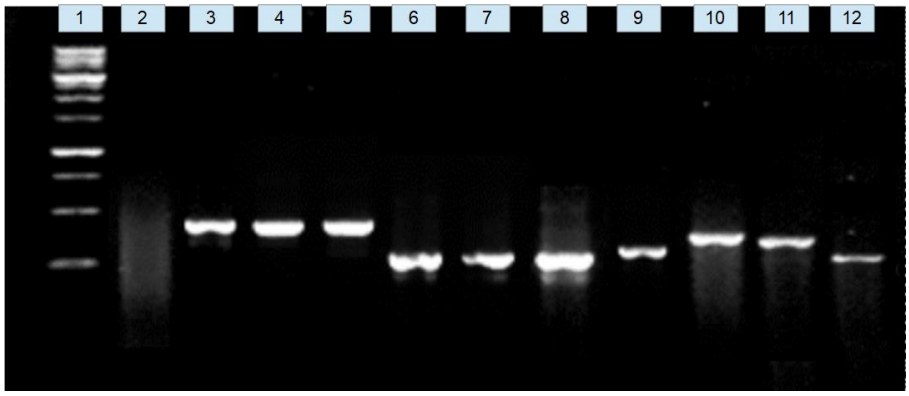

Figure 3. PCR detection of different *Enterococcus* species. Lane 1 100Kb size marker, 2 negative control, 3-5 *E. faecalis* (360bp), 6-8 *E. hirae* (187bp), 9 *E. faecium* (215bp), 10 *E. durans* (295bp), 11 *E. classeliflavus* (288 bp) and 12 *E. faecium* positive control.

Table 3. *Enterococcus* spp. composition and prevalence at different study sites.

		Source						
		Influent	Effluent	Sludge	Forest soil	El Mahsama drain	El Sayedeen lake	Total
Strain	*E. faecium*	21	18	24	17	18	18	116
	E. faecalis	14	9	14	11	11	11	70
	E. hirae	4	2	5	2	2	2	17
	E. durans	0	0	0	4	1	1	6
	E. casseliflavus	1	1	1	3	1	1	8
	Others	2	2	3	2	1	1	11
Total		42	32	47	39	34	34	228

Table 4. Antimicrobial susceptibility of *Enterococcus* spp. according to sampling sites.

	Influent		Effluent		Sludge		Forest soil		El Mahsama drain		El Sayedeen lake		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Tetracycline	27	21	16	13	28	22	20	16	18	14	19	15	128	56.14
Vancomycin	5	24	2	10	7	33	1	5	4	19	2	10	21	9.21
Ciprofloxacin	14	23	14	23	14	23	9	15	8	13	3	5	62	27.19
Erythromycin	25	20	17	14	27	22	19	16	18	15	16	13	122	53.51
Chloramphenicol	9	29	8	26	5	16	4	13	3	10	2	6	31	13.60
Nor	15	18	10	12	23	28	12	14	12	14	11	13	83	36.40
Rif	13	27	7	14	11	22	6	12	8	16	4	8	49	21.49
Amp	11	26	4	9	10	23	3	7	11	26	4	9	43	18.86
Total	42	18	32	14	47	21	39	17	34	15	34	15	228	100

Table 5. Average multiple antibiotic resistance (MAR) index per site and species

	E. faecium	*E. faecalis*	*E. hirae*	*E. durans*	*E. casseliflavus*	Other
Influent	0.393	0.384	0.281	0	0	0.25
Effluent	0.326	0.361	0.125	0	0	0.188
Sludge	0.370	0.312	0.3	0	0.25	0.208
Soil	0.287	0.261	0.188	0.125	0.125	0.063
Drain	0.319	0.318	0.25	0	0.125	0.375
Lake	0.236	0.284	0.063	0	0	0.125

Table 6. Numbers of different *Enterococcus* spp. for each MAR index per sampling source

Source	Species	0	0.125	0.25	0.375	0.5	0.625	0.75
Influent	*E. faecium*	0	5	4	2	6	1	3
	E. faecalis	0	2	1	6	4	1	0
	E. hirae	0	0	3	1	0	0	0
	E. durans	0	0	0	0	0	0	0
	E. casseliflavus	1	0	0	0	0	0	0
	Others	0	0	2	0	0	0	0
Effluent	*E. faecium*	0	1	8	6	3	0	0
	E. faecalis	0	1	2	3	3	0	0
	E. hirae	1	0	1	0	0	0	0
	E. durans	0	0	0	0	0	0	0

	E. casseliflavus	1	0	0	0	0	0	0
	Others	0	1	1	0	0	0	0
Sludge	*E. faecium*	0	3	8	4	7	2	0
	E. faecalis	0	5	2	3	3	1	0
	E. hirae	0	2	1	1	0	1	0
	E. durans	0	0	0	0	0	0	0
	E. casseliflavus	1	1	1	0	0	0	0
	Others	0	1	2	0	0	0	0
Soil	*E. faecium*	0	5	6	3	2	1	0
	E. faecalis	0	4	3	3	1	0	0
	E. hirae	0	1	1	0	0	0	0
	E. durans	0	4	0	0	0	0	0
	E. casseliflavus	1	0	0	0	0	0	0
	Others	1	1	0	0	0	0	0
Drain	*E. faecium*	1	2	6	5	3	1	0
	E. faecalis	0	2	3	4	2	0	0
	E. hirae	0	0	2	0	0	0	0
	E. durans	1	0	0	0	0	0	0
	E. casseliflavus	0	1	0	0	0	0	0
	Others	0	0	0	1	0	0	0
Lake	*E. faecium*	1	6	6	4	1	0	0
	E. faecalis	1	2	3	4	0	1	0
	E. hirae	1	1	0	0	0	0	0
	E. durans	1	0	0	0	0	0	0
	E. casseliflavus	1	0	0	0	0	0	0
	Others	0	1	0	0	0	0	0
	Total n (%)	13(5.7)	52(22.8)	66(28.9)	50(21.9)	35(15.3)	9(3.9)	3(1.3)

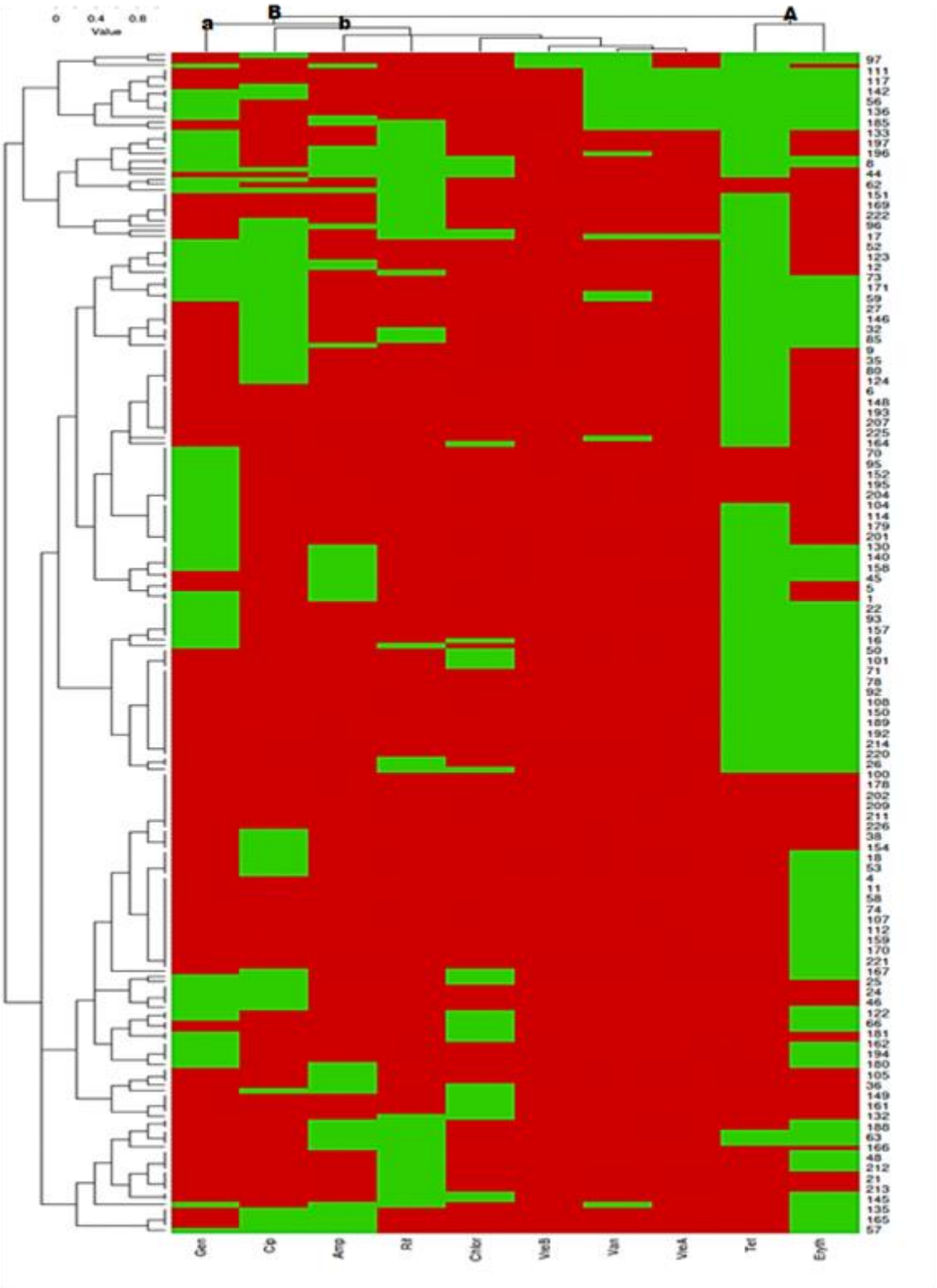

Figure 4. Heatmap and complete linkage cluster analysis for different strains in relation to antibiotic resistance, *van*A and *van*B and sampling sites. Each cell in a row represents a single susceptibility test result for a given *Enterococcus* spp. isolate. Green tiles represent resistance, orange tiles represent sensitive patterns. Strains isolated from the inlet (1:42), effluent (43:74), sludge (75:121), soil (122:160), ElHalous drain (161:194) and ElSayadeen lake (195:228).

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