Characterization of Antibiotics Resistant Enterococci Isolated from Epe Dam Water, Lagos Nigeria

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Abstract: Dams have been constructed worldwide to dependably provide people with safe water to drink, which has become the practice in developed areas of Nigeria and is acknowledged as a fundamental human right and a cost-effective measure for controlling disease. However, the global scientific community is increasingly concerned by descriptions of these dams to be aquatic reservoirs of antibiotic-resistant bacteria and/or genes. This study was aimed at the characterization of antibiotics resistant enterococci from Epe dam water in Lagos, Nigeria. Isolation was done by pour plating followed by streaking on selective agar. Isolated bacteria were identified using prescribed standard biochemical methods. Antibiotic susceptibility testing was done using the Kirby-Bauer disk diffusion technique against using clinically relevant antibiotics. The 66.66% of *Enterococcus faecalis*, 27.77% of *Enterococcus faecium*, and 5.57% of *Enterococcus gallinarum* were isolated from the samples. Isolates displayed varying high levels of resistance against test antibiotics. *Enterococcus faecalis* proved to be resistant to zinnacef (Z), amoxicillin (AM), rocephin (R), and streptomycin (S), *Enterococcus faecium* was resistant to zinnacef (Z), amoxicillin (AM), and streptomycin (SXT), while *Enterococcus gallinarum* is resistant to pefloxacin (PEF), gentamycin (GN), zinnacef (Z), amoxicillin (AM), and streptomycin (S). Hence, this study ascertains that dam waters are reservoirs of multiple antibiotics resistant Enterococci. Therefore, there is a need to improve hygiene conditions in the sampled water environment.
Key word: Dam water, antibiotics resistant, Enterococci, reservoir, isolates.

INTRODUCTION

Dams have been constructed worldwide to dependably provide people with safe water to drink, which has become the practice in developed areas of Nigeria and is acknowledged as a fundamental human right and an efficient way of preventing disease. Concerns about the microbial quality of water have traditionally been focused on the occurrence of pathogens and/or indicator bacteria (*Escherichia coli,* coliforms, enterococci) (Ashbolt *et al.*, 2001). However, the global scientific community is increasingly concerned by descriptions of aquatic reservoirs of antibiotic-resistant bacteria and/or genes. Both might be disseminated to humans and so contribute to the decrease of therapeutic alternatives and to the evolution and emergence of new genetic platforms with clinical consequences along with aquatic autochthon flora (Baquero *et al*., 2008; Martinez *et al*., 2009). The timely and better information on various aspects of water quality is considered an urgent requirement for both the general public and the drinking water industry.

Enterococci became the second leading cause of healthcare-acquired infections (Sievert *et al.,* 2013). Moreover, patients infected with enterococci have a high mortality rate of up to 61% (Fisher and Phillips, 2009). This is as a result of the persistent threat posed by enterococci antibiotic resistance which is of major concern (Calfee, 2012). Enterococci intrinsic resistance to a variety of antibiotics is common due to unique penicillin-binding proteins, enterococci can continue to synthesize their cell wall in the presence of β-lactam antibiotics, making some species of enterococci intrinsically resistant to penicillin, cephalosporins, and carbapenems (Moellering, 1992), while some enterococci such as *E. faecalis* is intrinsically resistant to macrolides, lincosamides, and streptogramin antibiotics (Dina *et al.*, 2003; Mazuski, 2008).

The dam water sources are out of official sanitization control and can be located in proximity to reservoirs of antibiotic-resistant

bacteria and genes (septic tanks, livestock yards, liquid-tight manure storage, and fertilizer storage). Although, the consumption of contaminated water should be considered a public health concern, untreated human drinking water is often an overlooked source in the epidemiology of antibiotic resistance in Epe Dam water. Data on antibiotic-resistant enterococci in the aquatic environment at this dam primarily pertains to surface waters and residual waters used for sporadic recreational activities.

An important characteristic of these microorganisms is their resistance to chemical and physical stress such as antibiotics and heavy metals in contrast to other faecal bacteria that are released into the environment (Choi *et al.*, 2003). Environmental sources may contribute to the dissemination of antibiotic-resistant enterococci (Junco *et al.*, 2001). The significant increases of multiple antibiotic-resistant (MAR) bacteria observed in various aquatic systems may be of significant importance to health since human infections caused by such organisms could be difficult to treat with drugs (Dicuonzo *et al.*, 2001).

The aim of this research is to isolate and identify Enterococci species in untreated water and investigate antibiotics susceptibility testing of the isolates so as to provide a deep understanding of antibiotic-resistant enterococci present in dam water.

MATERIALS AND METHODS

This study involves standard procedures of water samples collected, preserved, and transported to the microbiology laboratory of Olabisi Onabanjo University Ago-Iwoye, Nigeria to make sure the qualities between the collection points and laboratory identification and isolation of the microorganisms were not altered.

Sample processing: Different samples of water which are mainly used for human drinking and also used for animal drinking were collected from different regions in the Epe dam water, Epe, Nigeria. The exact timing of sample collection was determined according to sampling water for microbial analysis as previously described by Rice (2012). All samples are collected in the 5-liter sterile keg, Wide Mouth Environmental Sample Bottles, and then kept at 4°C in the cooler box packed with ice and transported to the laboratory for analysis within six hours.

Isolation of microorganisms: Six test tubes containing 9 ml of the diluents (distilled water) were prepared each for a total of 3 samples, then 1 ml of the initial suspension was withdrawn using a sterile pipette and then transferred into the first test tube to make a dilution of 10^{-1}. One ml was also withdrawn from the dilution of 10^{-1} and was transferred to the second test tube to make a dilution of 10^{-2}. This process continues for the third, fourth, fifth, and sixth test tubes to make a further dilution of 10^{-3}, $10^{-4,}$ and 10^{-5} respectively. This method was repeated for each sample to give a total of 40 test tubes prepared for the serial dilution. Serial dilution of 10^{-1}, $10^{-2,}$ and 10^{-3} was used for the enumeration of bacteria in the buns samples based on the microbial load.

The method used for the plating was the pour plate method in which samples were inoculated before pouring the medium into the Petri dishes. The Petri dishes labelled for each sample was then opened slightly and 1 ml of serial dilutions 10^{-1}, $10^{-2,}$ and 10^{-3} were then transferred using a pipette into their respective Petri dishes. The already prepared Eosin Methylene Blue Agar (EMB), MacConkey agar (MA), or Salmonella Shigella agar (SSA) was then poured into the plates containing 1 ml of the samples and gently swirled to ensure an even distribution of the medium. The setup was allowed to gel and incubated at 37°C for 24 hours.

After 24 hours of incubation, the different culture plates were examined for microbial growth. The total number of colonies present on the plate were counted which was within the range of 30-300 colony forming unit (cfu). The plate with colonies that are clumped together was then divided into 4 with the use of a ruler and a marker, and the number of distinct colonies was counted and

multiplied by four (4). The number of colonies counted was then multiplied by the dilution factor used and recorded as colony forming unit/ml (cfu/ml). The bacteria colony was differentiated based on the physical appearance on the plate, it was picked and streaked on a new nutrient plate for purification using a sterile inoculating loop and incubated at 35-37℃ for 24 hours. This process was done in order to have a distinct colony of bacteria growing on a nutrient agar plate. Each purified colony was then subcultured on the agar slant (Mac Cartney bottle) and incubated at 35-37℃ for storage at 4℃.

Microscopic and biochemical characterization of bacterial isolates: A smear of the pure isolate was made on a clean grease-free slide. The film was air–dried and heat-fixed by waving it over a Bunsen burner flame. The smear was covered with crystal violet reagent for one minute. This was rinsed in a slowly running tap for 5 seconds. The slide was covered with lugols iodine and allowed for 60 seconds. This was washed off in a slowly running tap. An alcohol reagent is used to decolourize the primary stain until no more dye runs off from the smear. The smear was covered with safranin reagent for 30 seconds. The slide was slowly rinsed under low running water. The glass is then air-dried and viewed under oil immersion lens microscope.

Catalase test: A colony of the bacteria from the culture plate was picked with a sterile glass rod and smeared into 3% hydrogen peroxide solution. Bubble formation was observed. This test is used to identify bacteria capable of producing the enzyme catalase, which breaks down hydrogen peroxide into water and oxygen. The reagent used is 3% hydrogen peroxide. A positive result is identified by the production of bubbles, which indicates oxygen production, and a negative result is identified by no bubble production (Chessbrough, 2010).

Oxidase test: A filter paper was placed in a Petri-dish and 3 drops of the oxidase reagent were added. Using a sterile glass rod, a colony of the bacteria cell was smeared on the filter paper. Colour change was observed. A negative result is identified by no colour change. This test is used to identify bacteria that produce the enzyme cytochrome oxidase, which catalyses the transport of electrons between electron donors and redox dye. The reagent used is tetramethyl-p-phenylenediamine hydrogen chloride (oxidase reagent). A positive result is identified by a purple coloration within 5-10 seconds and a negative result is identified by no colour change (Chessbrough, 2010).

Citrate utilization test: A slant of koser's Citrate agar was prepared in test tubes and allowed to solidify. The bacteria culture was then inoculated on the agar in the test tube slant and incubated for 24 h at 37ºC. This test is used to identify bacteria that can grow utilizing citrate as their sole carbon and energy source. A negative result is identified by green coloration on the media, and a positive result is identified by blue coloration (Chessbrough, 2010). False positive reaction was avoided by using wire loop that does not contain carbon deposits

Indole production test: A sterile inoculating loop was used to pick an inoculum from the culture plate of the bacteria. And then inoculated into a test tube containing peptone broth, and incubated for 24-48 h at 37ºC. After incubation, 5 drops of Kovac's reagent was added to it and colour change was observed. This test is used to identify bacteria capable of splitting indole from the amino acid tryptophan, using the enzyme tryptophanase. A positive result is identified by a red violet coloration and a negative result is identified by a yellow coloration (MacFaddin, 2000).

Motility test: The pure isolates in the stock culture were first inoculated into the nutrient broth to keep the organisms in an active growth phase. A hanging drop technique was carried out for motility. Drops of the cultures were placed on coverslips and plasticine was placed on slides enough to cover the coverslip. The slides were then gently placed over the drops of the cultures without allowing contact. The slides were

then gently placed over the drops of the cultures without allowing contact. The slides were quickly inverted, care being taken so that the drops from the coverslip do not touch the slide. These preparations were observed under the microscope. Motile organisms were seen moving from one corner of the coverslip to another while nonmotile organisms did not move about (Cheong *et al*., 2015).

Antibiotics susceptibility profile of the bacterial isolates from water samples: This test was performed with a panel of various commonly prescribed antibiotics using the disk diffusion method according to Clinical and Laboratory Standards Institute guidelines. An overnight culture of each isolate was prepared on nutrient broth and incubated at 37°C for 18 hours, dry sterile plates of prepared Mueller Hinton's agar was inoculated with the standardized inoculums of 18-hour culture test bacteria isolate. Thereafter, the minimum inhibitory concentration (mic) of the various antibiotics was determined by using agar plate dilution methods with some modifications and the lowest antibiotic concentration at which there was no visible growth was taken as the minimum inhibitory concentration (mic) (CLSI, 2015).

RESULTS

The result of Table 1 shows the total variable counts of bacterial isolates from Epe Dam water samples. The results show the colony growths on 3 selective agar; MacConkey agar, Salmonella-Shigella agar, and Eosine Methylene blue Agar. The serially diluted water samples plated on MacConkey agar, 7.2 × 10 cfu/ml was the highest counts while the lowest counts, was 2.8×10^4 cfu/ml. On Eosine Methylene Blue agar, 4.6×10^0 cfu/ml was the highest counts, while the lowest count was 1.1×10^{-2} cfu/ml. On Salmonella Shigella agar, 4.0 × 10 cfu/ml was the highest count while the lowest counts was 1.1×10^{-2} cfu/ml. The morphological characterization of the bacterial isolates from Epe Dam water shows the form, elevation, and colour. Most of the isolates were spherical in form and flat in elevation with red and brown being the most occurring colour. Isolate M1 to M6 were spherical as the physical form followed by the elevation which was flat and the colour which was red, Also, isolate E1 to E6, were spherical as the physical form, followed by the elevation which was flat and the colour which was brown/blue-black with the exception of E6 which was brown, and S1 to S6, were spherical as the physical form followed by the elevation which was flat and the colour which S1, S4, and S6 are pink/partially red, S2 and S3 are pink and S5 was originally partially pink. Also, the biochemical characterization of the isolates and the probable identities of the isolates. The probable organisms of M1 to E5 and S5 included *Enterococcus faecalis*, S1 TO S4 include *Enterococcus faecium* and E6 include *Enterococcus gallinarum*. All the isolates were non-motile with exception to E6 which was positive and they are catalase-negative. It was observed that all the isolates were oxidase-negative and indole-negative. The result in Table 2 below shows the percentage of occurrence of the probable microorganisms with *Enterococcus faecalis* having the highest occurrence at 66.66%, *Enterococcus faecium* with 27.77%, and *Enterococcus gallinarum* with 5.57%. Result in Table 3 below shows the antimicrobial susceptibility pattern of the probable bacteria in mm of the positive disk with *Enterococcus faecalis* which has the highest zone of inhibition of 32 mm on (APX) ampiclox disk, followed by (SXT) septrin 24 mm, (PEF) pefloxacin 21 mm, (CPX) ciprofloxacin 19 mm, (E) erythromycin 18 mm, (CN) gentamycin 15 mm, (S) streptomycin 12 mm, (R) rocephin 8 mm, (Z) zinnacef 7 mm and resistance to (AM) amoxicillin. *Enterococcus faecium* with the highest zone of inhibition on 28 mm (APX) ampiclox disk, followed by (S) streptomycin 25 mm, (E) erythromycin 22 mm, (CN) gentamycin 22 mm, (R) rocephin 22 mm, (CPX) ciprofloxacin 22 mm, (PEF) pefloxacin 18 mm, (Z) zinnacef 11 mm, (SXT) septrin 8 mm, and resistance to (AM)

amoxicillin. *Enterococcus gallinarum* with the highest zone of inhibition on 15 mm (E) erythromycin and (SXT) septrin disk, followed by (R) rocephin 13 mm, (APX) ampiclox and (CPX) ciprofloxacin 11 mm, (CN) gentamycin 8 mm, and resistance to (AM) amoxicillin, (PEF) pefloxacin, (Z) zinnacef and (S) streptomycin.

Table 1: Total variable counts, morphological characteristics, and biochemical characterization of bacterial isolates from the water samples

s/n	isolates code	dilution factors			MORPHOLOGICAL CHARACTERISTICS			BIOCHEMICAL CHARACTERIZATION							PROBABLE ORGANISM
		10^{-1}	10^{-2}	10^{-3}	Form	Elevation	Colour	Gram staining	Catalase Test	Oxidase test	Citrate test	Indole test	Motility test	Cellular morphology	
1	M1	69	50	39	SPH	Flat	Red	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
2	M2	64	44	28	SPH	Flat	Red	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
3	M3	67	48	35	SPH	Flat	Red	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
4	M4	72	54	48	SPH	Flat	Red	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
5	M5	68	48	41	SPH	Flat	Red	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
6	M6	69	50	33	SPH	Flat	Red	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
7	E1	39	26	18	SPH	Flat	BBb	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
8	E2	36	20	15	SPH	Flat	BBb	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
9	E3	30	17	11	SPH	Flat	BBb	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
10	E4	46	29	21	SPH	Flat	BBb	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
11	E5	37	21	12	SPH	Flat	BBb	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
12	E6	39	25	18	SPH	Flat	Brown	+ve	-ve	-ve	-ve	-ve	+ve	Cocci (spherical)	*Enterococcus gallinarum*
13	S1	32	21	15	SPH	Flat	PPr	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecium*
14	S2	35	23	12	SPH	Flat	Pink	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecium*
15	S3	30	16	11	SPH	Flat	Pink	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecium*
16	S4	38	26	18	SPH	Flat	PPr	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecium*
17	S5	40	29	19	SPH	Flat	Pr	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecalis*
18	S6	35	21	12	SPH	Flat	PPr	+ve	-ve	-ve	-ve	-ve	-ve	Cocci (spherical)	*Enterococcus faecium*

Key: +ve = Positive, -ve = Negative, SPH = Spherical, BBb = Brown and Blue-black, PPr = Pink and partially red, Pr = Partially red.

Table 2: Percentage occurrence of tested enterococci in water sample

S/N	Probable Organisms	Number of Occurrence	Percentage of Occurrence (%)
1	*Enterococcus faecalis*	12	66.66
2	*Enterococcus faecium*	5	27.77
3	*Enterococcus gallinarum*	1	5.57
	Total	**18**	**100%**

DISCUSSION

Faecal enterococci are standard indicators of faecal contamination in aquatic environments and more specifically *Enterococcus. faecalis* is now considered as the indicator of choice for recreational waters (Lleò *et al.*, 2005). Among the 24 enterococcal species known so far, *E. faecalis* and *E. faecium* are the most important ones responsible for infections in humans (Klare *et al*., 2003). Coastal areas contaminated with faecal effluents constitute a significant risk to public health (Borrego and José Figueras, 1997). Epidemiological studies have revealed that there is a correlation between microbiological water quality and swimming-associated illness, and faecal streptococci are the causative agent in diseases such as gastrointestinal and respiratory system and skin illness (Fleisher *et al*., 1996).

Table 3: Antimicrobial susceptibility pattern of bacterial isolates (mm) from the sample water

S/N	probable organisms	positive disk (+ve)									
		E (10µg)	PEF (10µg)	CN (10µg)	APX (30µg)	Z (20µg)	AM (30µg)	R (25µg)	CPX (10µg)	S (30µg)	SXT (30µg)
1	*Enterococcus faecalis*	18	21	15	32	7	R	8	19	12	24
2	*Enterococcus faecium*	22	18	22	28	11	R	22	22	25	8
3	*Enterococcus gallinarum*	15	R	8	11	R	R	13	11	R	15

Keys: S/N: serial number, E: Erythromycin, PEF: Pefloxacin, APX: Ampiclox, CN: Gentamycin, Z: Zinnacef, AM: Amoxicillin, R: Rocephin, CPX: Ciprofloxacin, S: Streptomycin, SXT: Septrin.

Table 4.4. Antimicrobial susceptibility test pattern of bacterial isolates (mm) from the sample water

S/N	Isolate	NEGATIVE DISC									
		SP (10µg)	PEF (10µg)	CH (30µg)	CN (10µg)	OFX (10µg)	AM (30µg)	AU (30µg)	CPX (10µg)	S (30µg)	SXT (30µg)
1	*Enterococcus faecalis*	23	8	18	32	24	R	14	14	R	28
2	*Enterococcus faecium*	15	23	22	16	18	R	11	22	R	13
3	*Enterococcus gallinarum*	8	11	8	4	11	R	R	11	R	18

Keys: S/N: serial number, CN: Gentamycin, AM: Amoxicillin, CPX: Ciprofloxacin, S: Streptomycin, AU: Augmentin, SP: Sparfloxacin, PEF: Pefloxacin, CH: Chloramphenicol, OFX: Tarivid, SXT: Septrin.

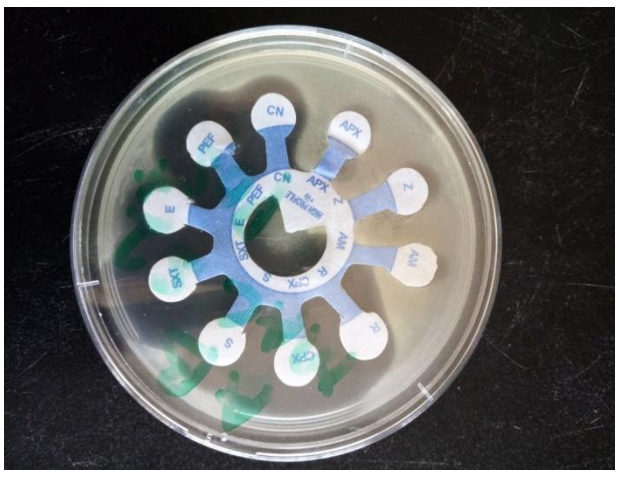

Plate 1: Zone of inhibition of *Enterococcus faecalis* present on positive disk plate using the disk plate method

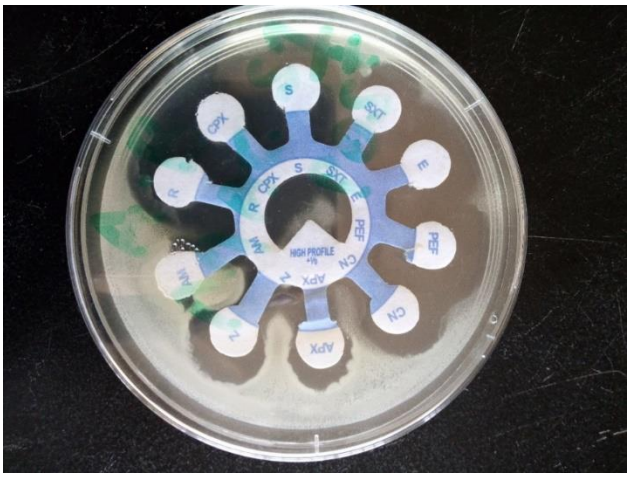

Plate 2: Zone of inhibition of *Enterococcus faecium* present on positive disk plate using the disk plate method

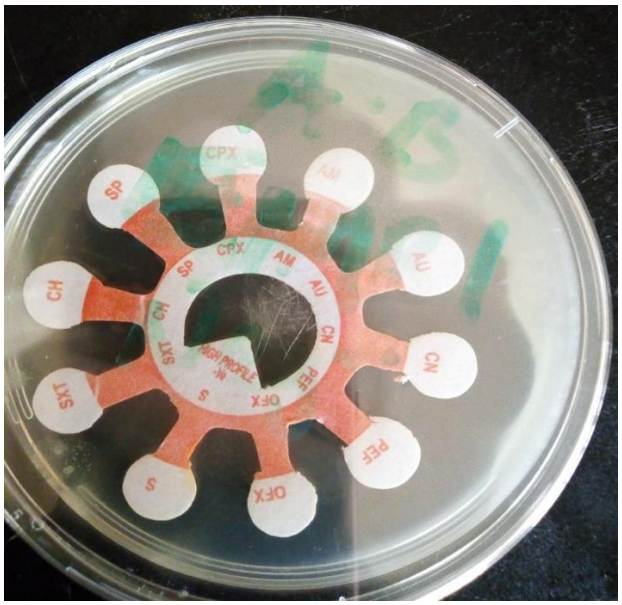

Plate 3: Zone of inhibition of *Enterococcus faecium* present on negative disk plate using the disk plate method

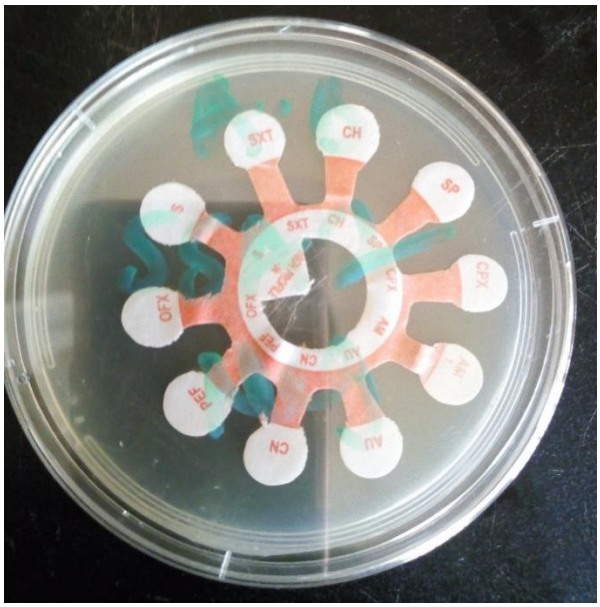

Plate 4: Zone of inhibition of *Enterococcus faecium* present on negative disk plate using the disk plate method

There are a number of studies related to the isolation of clinical and environmental samples of faecal streptococci (Kacmaz and Aksoy, 2005). In these studies, it was reported that *E. faecalis* is a strain isolated frequently (Junco *et al.*, 2001). Moreover, *E. faecalis* is the predominant species both in environmental and clinical samples (Moaddab and Toreci, 2000). In the present study, the isolates mainly consisted of *E. faecalis* because the coastal areas were likely mpacted by the discharges of seagulls and humans in which *E. faecalis* was predominant. This is in agreement with the findings of Pourcher *et al*. (1991) who found that *E. faecalis* was prominent in humans and seagulls. The amount of *E. faecalis* isolated was similar to that detected in environmental samples by other authors (Genthner *et al.,* 2005).

Based on the findings, *Enterococcus faecium* (27.77%) and *Enterococcus gallinanum* (5.57%) were also isolated from the samples, although the isolation of *Enterococcus gallinanum* in environmental isolates was not reported before (Genthner *et al.,* 2005) *Enterococcus. faecalis* was frequently isolated as predominant species in this study and other studies. It is now clear that the isolation of these bacteria might be used as evidence of faecal contamination in water samples, and, *E. faecalis* is now considered the indicator of choice for untreated waters.

In this study, all antibiotic discs were tested by the disc diffusion method using a separated disk (Positive disk and Negative disk). Among the strains, 2 and 6% were resistant to amoxicillin and streptomycin, respectively. *Enterococcus faecalis* susceptibility to amoxicillin is of great importance since amoxicillin is the drug of choice in the treatment of enterococcal infections (Barisic and Punda-Polić, 2000). The various rates of amoxicillin and streptomycin resistance in both clinical (Lopes *et al.,* 2005) and environmental isolates (Junco *et al.,* 2001) were previously reported. Kacmaz and Aksoy. (2005) showed that of the 207 *E. faecalis* strains isolated from clinical specimens, 12% were resistant to amoxicillin while 10% were resistant to streptomycin. Lopes *et al.* (2005) found that all *E. faecalis* strains isolated were sensitive to amoxicillin. Among the strains isolated in this study, 6% were resistant to amoxicillin,

while none of the strains isolated were resistant to amoxicillin and this is in agreement with the report of Junco *et al.* (2001).

The majority of enterococcal resistances to this class of antibiotics were due to the modifications in the amoxicillin-binding proteins (ABPs) (Klare *et al.*, 2003). From the findings of other studies as well as the current study, it could be suggested that both amoxicillin and streptomycin discs should be evaluated simultaneously by the disc diffusion method, to detect the resistance of enterococci to penicillin group antibiotics (Kacmaz and Aksoy, 2005).

CONCLUSION

This study revealed that testing for antibiotic and heavy metal susceptibility on environmental bacterial strains could aid in conducting epidemiological research, identifying the part these strains play in the spread of antibiotic and heavy metal resistance, and monitoring a wide range of harmful pollutants in environments contaminated with enterococci. This present work in this context adds new knowledge on the occurrence of antibiotic-resistant enterococci in untreated human drinking water from developed towns and cities. It demonstrates that antibiotic-resistant bacteria are contaminating Epe natural, untreated water sources, most likely from the roosts of humans and other animal niches that surround the water collection spots.

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