Antibiogram signatures of non-cholera causing *Vibrio* species recovered from environmental niches in Eastern Cape, South Africa.

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Research Article

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Abstract

Background: The use of antibiotics globally has helped reduce mortality and morbidity rate due to its ability to effectively treat bacterial infections in both humans and animals. However, the menace of antimicrobial resistance has become a challenge to public health due to its increased mortality and morbidity rate. This study determined the antibiogram pattern of non-cholera causing *Vibrio* species against a panel of 11 antibiotics that are wildly used for treatment. Multiple antibiotic resistance phenotype, multiple antibiotic resistant indices and minimum inhibitory concentration (MIC) of test antibiotics were also determined.

Results: Polymerase chain reaction (PCR) was used to confirm 100 isolates of *Vibrio parahaemolyticus*, 82 and 46 isolates of *Vibrio vulnificus* and *Vibrio fluvialis* respectively, collected from the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare. Thereafter, disc diffusion method was used to determine the antibiogram pattern of target non-cholera causing *Vibrio* species against a panel of 11 antibiotics that are of clinical importance. The highest rate of *Vibrio parahaemolyticus* resistance was observed against tetracycline (22 %) and nalidixic acid (16 %). *Vibrio fluvialis* also displayed highest rate of resistance against tetracycline (28 %) and nalidixic acid (28 %), while *Vibrio vulnificus* isolates exhibited highest rate resistance against imipenem (40 %) and tetracycline (22 %). A total of 38 MARP patterns were observed and the MAR indices ranged between 0.3 and 0.8. Against the resistant *Vibrio parahaemolyticus* and *Vibrio fluvialis* isolates, minimum inhibitory concentration ranged from 16 µg/ml to 2048 µg/ml for both tetracycline and nalidixic acid, while against *Vibrio vulnificus* isolates, minimum inhibitory concentration ranged from 8 µg/ml to 256 µg/ml for both imipenem and nalidixic acid.

Conclusions: Results obtained from this study is an indication that antibiotic resistant bacteria that could pose as threat to health of humans and animals are present in the environment.

1. Background

Vibrio species are ubiquitous organisms that are highly abundant in aquatic environment. Most species are pathogenic causing mild to severe infections in humans, especially patients with underlying health conditions [11]. The isolation and distribution of *Vibrio* species have been reported by several studies globally as infections caused by this group of bacteria is still a threat to public health. *Vibrio* infections occur when contaminated seafood or water is consumed [22]. The use of antibiotics globally has helped reduce mortality and morbidity rate due to its ability to effectively treat bacterial infections in both humans and animals [28]. Antibiotics such as tetracycline, cephalosporin, fluoroquinolones, aminoglycosides, and imipenem are some of the clinically acceptable antibiotics for treatment of *Vibrio* infections [5]. However, the development of resistance to these antibiotics is an increasing worldwide challenge due to its association with increased mortality and morbidity rate [13]. Extensive and inappropriate use of antibiotics are factors contributing to the emergence and spread of antibiotic resistance in the environment [1].

Antimicrobial resistance is not a recent challenge in the world today, but the number of resistant organisms, the geographical environment affected by antimicrobial resistance and the prevalence of resistance in organisms are exceptional and increasing drastically [21]. *Vibrio* species are usually susceptible to most antibiotics that are of clinical importance, but several studies have recorded resistance to some of these antibiotics due to their excessive usage in humans and animals, agriculture and aquaculture system [12]. In South Africa, consumption of contaminated water is a key source of *Vibrio* infection as most people rely solely on surface water as their main source of water for various day-to-say activities [31]. Over the years, studies have reported surface water to be reservoirs of antimicrobial resistant bacteria and this is due to different anthropogenic activities and release of improperly treated wastewater effluent into the environment [9]. The studies of [3, 15, 16, 17, 25, 37, 41] reported increased rate of resistance in *Vibrio* species against antibiotics that are recommended for treatment. Despite the high rate of resistance of *Vibrio* species to these recommended drugs for treatment, some other studies have also reported high susceptibility rate of *Vibrio* species against these drugs [18, 29, 36]. This study therefore, aims to determine the antibiogram profiling of non-cholera causing *Vibrio* species recovered from environmental niches in Eastern Cape, South Africa.

2. Results

2.1. Molecular confirmation of Vibrio species

A total of 228 isolates belonging to the genus *Vibrio* were obtained from the archive collection of AEMREG. One hundred isolates of *Vibrio parahaemolyticus* were confirmed using a 503 base pair gene marker as shown in Fig. 1. Eighty-two isolates were *Vibrio vulnificus* using a 410 base pair gene marker as shown in Fig. 2. The remaining 46 isolates were *Vibrio fluvialis* using a 217 base pair gene marker shown in Fig. 3.

Figure 1: A gel electrophoresis picture showing the confirmation of *Vibrio parahaemolyticus* using *toxR* gene. Lane MM: 100 base pair gene marker; lane 1: Positive control (DSM 10027); lane 2: Negative control; lane 3 to 12: Positive isolates.

2.2. Antibiotic susceptibility profiling of Vibrio species

All confirmed 228 *Vibrio* isolates were subjected to antibiotic susceptibility testing. The highest rate of resistance in *Vibrio parahaemolyticus* was recorded against Tetracycline (22 %). For *Vibrio vulnificus*, the highest rate of resistance was observed against imipenem (40 %), while for *Vibrio fluvialis*, the highest rate of resistance was observed against Nalidixic acid (28 %) and tetracycline (28 %). The summary of the results are presented in Table 1.

Table 1
Antibiotic susceptibility pattern of target *Vibrio* species.

Group	Antibiotic (µg)	*Vibrio parahaemolyticus* n = 100 (frequency/percent)			*Vibrio vulnificus* n = 82 (frequency/percent)			*Vibrio fluvialis* n = 46 (frequency/percent)		
		S	I	R	S	I	R	S	I	R
Carbapenems	Imipenem (10)	98/98	2/2	0	31/38	18/22	33/40	39/ 85	7/15	0
	Meropenem (10)	96/96	4/4	0	64/78	6/7	12/15	46/100	0	0
Penicillins	Ampicillin (10)	82/82	4/4	14/14	57/70	10/12	15/18	35/76	3/7	8/20
	Augmentin (20/10)	90/90	4/4	6/6	79/96	0	3/4	39/85	5/11	2/4
Cephems	Cefotaxime (30)	74/74	12/12	14/14	68/83	9/11	5/6	38/83	3/6	5/11
Aminoglycosides	Amikacin (30)	87/87	2/2	11/11	77/94	4/5	1/1	32/70	9/19	5/11
Fluoroquinolones	Ciprofloxacin (5)	88/88	8/8	4/4	68/83	12/15	2/2	38/83	6/13	2/4
	Nalidixic acid (30)	78/78	6/6	16/16	60/73	16/19	7/9	29/63	4/9	13/28
Phenicols	Chloramphenicol (30)	80/80	8/8	12/12	53/65	20/24	9/11	30/65	9/20	7/15
Tetracycline	Tetracycline (30)	69/69	9/9	22/22	59/72	4/5	18/22	32/70	½	13/28
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole (1.25/23.75)	92/92	1/1	7/7	73/89	9/11	0	41/89	½	4/9

2.3. Pattern of MAR phenotypes and MAR index of Vibrio species

A total of 38 multiple antibiotic resistance phenotype patterns were observed for *Vibrio* species evaluated in this study. Approximately 23% of the *Vibrio* isolates exhibited resistance to three or more antibiotics. The multiple antibiotic resistance

index of the isolates was observed to be greater than 0.2 with the highest being 0.8 and lowest being 0.3. Summary of the result is presented in Table 2.

Table 2
Multiple antibiotic resistance phenotypes and multiple antibiotic resistance index of *Vibrio* species.

Name of *Vibrio* specie	Number of isolates with same resistant pattern	Antibiotic resistance pattern	MARP	MARI
Vibrio parahaemolyticus	1	AP-CTC-AK-T-NA-CIP-AG-C-TS	9	0.8
	1	AG-T-NA-TS	4	0.4
	1	AK-AP-C-T	4	0.4
	1	AG-T-NA	3	0.3
	1	AP-AG-AK-T-CIP-NA-TS	7	0.6
	1	T-AP-C-CTX	4	0.4
	1	CTX-AP-T-NA	4	0.4
	1	AK-CTX-AP-NA-T	5	0.45
	1	T-AK-NA	3	0.3
	1	AK-AP-CTX-C	4	0.4
	1	AK-AP-CTX-NA-T-TS	6	0.55
	1	T-AK-NA-TS	4	0.4
	1	CTX-AP-C-T-TS-NA	6	0.55
	2	AP-CTX-AK-T-CIP-AG-C-NA	8	0.7
	2	T-TS-NA	3	0.3
	2	AK-NA-CTX-C-T	5	0.45
	3	CTX-AP-T-C	4	0.4
Vibrio vulnificus	1	IMI-MEM-AP-CTX-T	5	0.45
	1	IMI-MEM-AP-CP-T-AK-C-CTX	8	0.7
	1	IMI-NA-C	3	0.3
	1	IMI-CIP-AP-NA-T	5	0.45
	1	IMI-AG-NA-AP	4	0.4
	1	IMI-MEM-CTX-T-C	5	0.45
	1	AP-IMI-MEM-CTX-T-C	6	0.55
	2	IMI-T-C	3	0.3
	2	AP-IMI-MEM-T	4	0.4
	3	IMI-T-AP	3	0.3
	3	IMI-MEM-AP-T-C-NA	6	0.55
Vibrio fluvialis	1	AP-T-NA-AG	4	0.4
	1	T-NA-AK-AP-C	5	0.45
	1	T-AP-AK-CIP-NA-CTX	6	0.55

Legend: AG-Augmentin; AP-Ampicillin; AK-Amikacin; C-Chloramphenicol; CIP-Ciprofloxacin; CTX-Cefotaxime; IMI-Imipenem; MEM-Meropenem; NA-Nalidixic acid; T-Tetracycline; TS-Trimethoprim-sulfamethoxazole.

Name of *Vibrio* specie	Number of isolates with same resistant pattern	Antibiotic resistance pattern	MARP	MARI
	1	T-AG-AP-NA	4	0.4
	1	T-AK-C-NA	4	0.4
	1	T-C-NA	3	0.3
	1	CIP-T-TS-NA	4	0.4
	1	T-CTX-C-NA-AP	5	0.45
	2	T-AK-NA	3	0.3
	3	T-CTX-TS-NA-C-AP	6	0.55

Legend: AG-Augmentin; AP-Ampicillin; AK-Amikacin; C-Chloramphenicol; CIP-Ciprofloxacin; CTX-Cefotaxime; IMI-Imipenem; MEM-Meropenem; NA-Nalidixic acid; T-Tetracycline; TS-Trimethoprim-sulfamethoxazole.

2.4. Minimum inhibitory concentration (MIC) of the antimicrobial agents.

The minimum inhibitory concentration (MICs) of the antibiotics against which the *Vibrio* isolates exhibited highest rate of resistance were determined. As observed in Tables 1 and 2, tetracycline and nalidixic acid are the most common antibiotics against which *Vibrio parahaemolyticus* and *Vibrio fluvialis* are resistant against. While imipenem and tetracycline appear to be the most common antibiotics *Vibrio vulnificus* is resistant against as shown. Against *Vibrio parahaemolyticus* and *Vibrio fluvialis* the MIC of tetracycline and nalidixic acid ranged from 16 µg/ml to 2048 µg/ml respectively, while in *Vibrio vulnificus* MIC of Imipenem and tetracycline ranged from 8 µg/ml to 256 µg/ml. Summary of the MIC results are presented in Tables 3, 4 and 5.

Table 3
Minimum inhibitory concentration (MIC) of selected antibiotics against *Vibrio parahaemolyticus*.

Antimicrobial agents	No. of resistant isolates	MIC concentration range (µg/ml)									
		4	8	16	32	64	128	256	512	1024	2048
Nalidixic acid	16	-	-	2	2	2	4	3	2	1	-
Tetracycline	16	-	-	1	7	3	5	-	-	-	-

Table 4
Minimum inhibitory concentration (MIC) of selected antibiotics against *Vibrio vulnificus*.

Antimicrobial agents	No. of resistant isolates	MIC concentration range (µg/ml)									
		1	2	4	8	16	32	64	128	256	512
Imipenem	16	-	-	-	2	6	5	3	-	-	-
Tetracycline	16	-	-	-	-	-	2	9	4	1	-

Table 5
Minimum inhibitory concentration (MIC) of selected antibiotics against *Vibrio fluvialis*.

Antimicrobial agents	No. of resistant isolates	MIC concentration range (µg/ml)									
		4	8	16	32	64	128	256	512	1024	2048
Tetracycline	13	-	-	-	-	-	-	5	4	3	1
Nalidixic acid	13	-	-	-	1	-	1	1	3	1	6

2.5. Minimum bactericidal concentration (MBC) of the antimicrobial agents

Minimum bactericidal concentration (MBC) was carried out on all the resistant *Vibrio* species starting with antibiotic concentrations that inhibited bacteria growth. At higher concentrations ranging from 256 µg/ml to 4096 µg/ml, nalidixic acid and tetracycline eliminated all the resistant *Vibrio parahaemolyticus* species. Against *Vibrio fluvialis*, due to high resistance observed against the test antibiotics (nalidixic acid and tetracycline), the antibiotics concentration was increased. At 8192 µg/ml, tetracycline and nalidixic acid eliminated 46.2 % and 69.2 % of the *Vibrio fluvialis* isolates respectively. At concentrations ranging from 32 µg/ml to 1024 µg/ml, imipenem had a bactericidal effect on *Vibrio vulnificus*, while, tetracycline had MBC values ranging from 256 µg/ml to 4096 µg/ml. Summary of the results are expressed in Tables 6, 7 and 8.

Table 6
Minimum bactericidal concentration (MBC) of test antibiotics against *Vibrio parahaemolyticus*.

Antimicrobial agents	No. of resistant isolates	MBC concentration range (µg/ml)							
		32	64	128	256	512	1024	2048	4096
Nalidixic acid	16	-	-	-	1	-	4	3	8
Tetracycline	16	-	-	-	-	3	5	6	2

Table 7
Minimum bactericidal concentration (MBC) of test antibiotics against *Vibrio vulnificus*.

Antimicrobial agents	No. of resistant isolates	MBC concentration range (µg/ml)							
		32	64	128	256	512	1024	2048	4096
Imipenem	16	1	-	5	3	5	2	-	-
Tetracycline	16	-	-	-	2	4	7	2	1

Table 8
Minimum bactericidal concentration (MBC) of test antibiotics against *Vibrio fluvialis*.

Antimicrobial agents	No. of resistant isolates	MBC concentration range (µg/ml)							
		64	128	256	512	1024	2048	4096	8192
Tetracycline	13	-	-	-	-	1	-	5	6
Nalidixic acid	13	-	-	-	-	-	1	3	9

3. Discussion

The isolation of *Vibrio* species from the environment is an indication that other human pathogenic microorganisms are present in the environment. This could be due to environmental contamination resulting from human activities or the discharge of untreated wastewater final effluent into the environment. In this study, PCR was used for the confirmation of *Vibrio* species. These confirmed *Vibrio* species were subjected to antibiotics susceptibility testing. The results observed show that *Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio fluvialis*) expressed resistance against almost all the antibiotics tested in this study. Resistance against antibiotics is an important medical and public health issue of concern due to its direct link with disease management [34]. Most of the *Vibrio* isolates were resistant against more than three antibiotics tested. Twenty-nine percent of the *Vibrio* isolates exhibited resistance against tetracycline, while 71 % of the isolates were susceptible against tetracycline. This report corroborates with the reports of Quilici *et al*. [33]; Raissy *et al*. [35]; Osuolale and Okoh, [32].

In contrast to this report, Mandal *et al*. [23] and Singh *et al*. [38] reported that *Vibrio* species exhibited a high resistance rate against tetracycline. Against nalidixic acid, 27 % of the *Vibrio* isolates were resistant, and 73 % were susceptible. This is in contrast to the findings of Srinivasan *et al*. [39], who reported that 73 % of *Vibrio* species were resistant against nalidixic acid. High rate of resistance against nalidixic acid is very concerning, as reports have stated that bacteria resistance to nalidixic acid is most likely to spread to other fluoroquinolones [20, 26]. No resistance pattern was observed in *Vibrio parahaemolyticus* and *Vibrio fluvialis* against imipenem and meropenem, while in *Vibrio vulnificus*, no resistance was observed against Trimethoprim-sulfamethoxazole. Baron *et al*. [4] reported increased Vibrio species' susceptibility to imipenem, ampicillin, amikacin and trimethoprim-sulfamethoxazole. This study is similar to that finding except for imipenem to which *Vibrio vulnificus* exhibited resistance. Okoh and Igbinosa, [30] also reported resistance to these antibiotics, including Imipenem and *Vibrio vulnificus* in this study exhibited resistance to imipenem, thereby corroborating with this report.

The multiple antibiotic resistance phenotype (MARP) evaluated in this study revealed that 38 MARP patterns were observed across all *Vibrio* species evaluated and most of the *Vibrio* isolates were resistant to three or more antibiotics, therefore, indicating that the isolates are resistant to almost all clinically important antibiotics used for treatment. The multiple antibiotic resistance index (MARI) index of 0.2 is the acceptable threshold values for differentiating low-risk and high-risk antibiotic usage regions. MAR index observed in this study ranged from 0.3 to 0.8 and this can be grouped under high-risk source of contamination. None of the isolates tested had MAR index value of ≤ 0.2. This therefore indicate inappropriate antibiotics usage in the environment. Increased MARI value like the one observed in this study could be as a result of various anthropogenic activities within the environment, thus suggesting that the environment is highly polluted with antimicrobial agents [2]. The MARI value obtained also confirms the findings of Okoh and Igbinosa [30], whose findings also revealed ≥ 0.3 threshold value. Resistance to tetracycline and nalidixic acid were the most dominant antibiotics to which *Vibrio parahaemolyticus* (16 isolates) and *Vibrio fluvialis* (13 isolates) are resistant against. While tetracycline and imipenem were the most common antibiotics to which *Vibrio vulnificus* (16 isolates) are resistant. Hence, Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was carried out using two antibiotics to which highest rate of resistance was observed.

As shown in Table 3, against *Vibrio parahaemolyticus*, MIC concentration ranged from 16 µg/ml to 1024 µg/ml in nalidixic acid and 16 µg/ml to 128 µg/ml in tetracycline. At 128 µg/ml concentration, nalidixic acid had the highest number of bacteria inhibitions, where 4 (25 %) out of the 16 resistant *Vibrio parahaemolyticus* isolates tested were inhibited. Antibiotics concentration 1024 µg/ml recorded the lowest number of inhibitions where only 1 (6 %) of the 16 resistant isolates was inhibited. Tetracycline recorded the highest number of inhibitions at 32 µg/ml, where 7 (43.8 %) out of the 16 resistant *Vibrio parahaemolyticus* isolates were inhibited. MBC was further carried out and in Table 6, at 2048 µg/ml and 4096 µg/ml concentrations of tetracycline and nalidixic acid respectively, most of the isolates were completely eliminated. These concentrations were then taken as the MBC values for both antibiotics against *Vibrio parahaemolyticus*.

In Table 4, against *Vibrio vulnificus*, MIC concentration observed ranged from 8 µg/ml to 64 µg/ml for imipenem and 32 µg/ml to 256 µg/ml for tetracycline. Imipenem had the highest number of bacteria growth inhibition at 16 µg/ml concentration, where 6 (37.5 %) of the resistant isolates were inhibited. The highest number of bacteria inhibitions for tetracycline was observed at 64 µg/ml concentration, where 9 (56.3 %) of the 16 resistant isolates were inhibited. At concentration 8 µg/ml and 256 µg/ml for imipenem and tetracycline respectively, lowest number of bacteria inhibitions was observed. MBC was carried out against the resistant *Vibrio vulnificus* isolates. 128 µg/ml and 512 µg/ml concentration of imipenem exhibited highest rate of bactericidal

activity, while 1024 µg/ml concentration of tetracycline exhibited highest number of bactericidal activities as shown in Table 7. These concentrations were taken as the MBC values for both antibiotics.

MIC concentration against *Vibrio fluvialis* ranged from 256 µg/ml to 2048 µg/ml for tetracycline and 32 µg/ml to 2048 µg/ml for nalidixic acid. Tetracycline and nalidixic acid had the highest number of bacteria growth inhibition at 256 µg/ml and 2048 µg/ml concentration respectively, where 5 (38.5 %) and 6 (46.2%) of the resistant isolates were inhibited by tetracycline and nalidixic acid respectively as shown in Table 5. MBC was carried out against the resistant *Vibrio fluvialis* isolates and at 8192 µg/ml concentration, tetracycline and nalidixic exhibited highest number of bactericidal activities. These concentrations were taken as the MBC values for both antibiotics. The MIC and MBC result in this study is in agreement with the result obtained from the disc diffusion susceptibility testing as resistance to tetracycline, nalidixic acid and imipenem were recorded in both tests. The report of Chandrakala *et al*. [8] is in contrast with findings from this present study as increased sensitivity of *Vibrio* species against tetracycline and nalidixic acid with low MIC values were observed.

4. Conclusions

Excessive and inappropriate use of antibiotics has contributed greatly to the development and spread of antimicrobial resistance. The isolation of *Vibrio* species from the environment is an indication that other pathogenic organisms that could pose as threat to health of humans and animals are present in the environment. This therefore, suggest that the environment is a potential source of resistant organisms. The MIC and MBC results revealed that at higher concentrations, antibiotics that are considered bacteriostatic can be bactericidal. Therefore, there is need for continuous monitoring and control of drug usage in the environment to ensure effective and appropriate treatment of infections. Environmental hygiene should also be practiced by members of the community to prevent outbreak of infections.

5. Materials And Methods

5.1. Sample collection

A total of 228 *Vibrio* isolates used in this research were collected from the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare. The organisms were originally isolated from various environmental niches in Eastern Cape, South Africa.

5.2. DNA extraction

Boiling method was used for the extraction of bacterial DNA as describe by Maugeri *et al*. [24]. The *Vibrio* isolates stored in glycerol stock at -80 ºC were resuscitated by inoculating into sterile Nutrient broth and incubated at 37 ºC for 24 h. After incubation, a loopful from the nutrient broth culture was suspended in 200 µl sterile distilled water, vortexed and boiled on a heating block for 15 min at 100 ºC in other to lyse the cell. The cell suspension was allowed to cool before been centrifuged at 15,000 rpm for 5 min. Thereafter, the supernatant was used as DNA template in polymerase chain reaction (PCR) assay.

5.3. Molecular confirmation of target Vibrio species

Polymerase chain reactions (PCR) was used to confirm the identities of the target *Vibrio* species using specific primers and cycling conditions described in Table 9. A 25µl final reaction mixture was used consisting of 12.5µl master mix, 5µl of DNA template, 0.5µl each of both forward and reverse primer and 6.5µl of nuclease-free (Fri *et al*., 2017). The PCR products were further resolved in a 1.5 % agarose gel stained with ethidium bromide for 45 min at 100 volts. A UV trans-illuminator (ALLIANCE 4.7) was used to visualize and photograph the resolved PCR product.

Table 9
Primer set and cycling conditions used for the confirmation of *Vibrio* species

Target strain	Primer	Sequence (5'–3')	PCR cycling condition	Product Size(bp)	References
Vibrio parahaemolyticus	tox r F tox r R	TGTACTGTTGAACGCCTAA CACGTTCTCATACGAGTG	Initial denaturation: 94°C (5 min), 35 cycles of {Denaturation: 94°C (30 sec), Annealing: 55°C (30 sec), Elongation: 72°C (30 sec)} and Final extension: 72°C (10 min).	503	[27]
Vibrio vulnificus	VvhA F VvhA R	ACTCAACTATCGTGCACG ACACTGTTCGACTGTGAG	Initial denaturation: 94°C (5 min), 35 cycles of {Denaturation: 94°C (30 sec), Annealing: 55°C (30 sec), Elongation: 72°C (30 sec)} and Final extension: 72°C (10 min).	410	[27]
Vibrio fluvialis	toxR F toxR R	GGATACGGCACTTGAGTAAGACTC GACCAGGGCTTTGAGGTGGACGAC	Initial denaturation: 94°C (5 min), 30 cycles of {Denaturation: 94°C (30 sec), Annealing: 57°C (1 min), Elongation: 72°C (90 sec)} and Final extension 72°C (7 min).	217	[7]

5.4. Antibiotics susceptibility testing of the confirmed Vibrio species

Confirmed isolates of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio fluvialis* were subjected to antibiotics susceptibility test using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar as described by CLSI [6]. Colonies from a pure culture grown overnight were transferred into 5ml sterile normal saline to adjust the turbidity to 0.5 McFarland standards. Sterile swab stick was used to spread the bacteria suspension uniformly on Mueller-Hinton agar and antibiotic discs were impregnated on the plate. The plates were incubated for 24 h at 37 ºC. Afterward, zones of inhibition were measured and the results were classified as resistant, intermediate and susceptible. A panel of 11 antibiotics often used for the treatment of infections caused by *Vibrio* species were used and they include: ampicillin (10 µg), augmentin (30 µg), cefotaxime (30 µg), imipenem (10 µg), meropenem (10 µg), tetracycline (30 µg), amikacin (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg) and trimethoprim-sulfamethoxazole (25 µg). Due to the unavailability of breakpoint zone diameter for *Vibrio* species by CLSI [6], the interpretative zone diameter of *Enterobacteriaceae* was used.

5.5. Multiple antibiotic resistance phenotypes (MARPs) and multiple antibiotic resistance index (MARI)

Multiple antibiotic resistance phenotype (MARP) was generated for isolates that exhibited resistance to three or more antibiotics as described by Titilawo *et al*. [42]. Multiple antibiotic resistance index (MARI) as described by Krumperman, [19], was determined using the formula: $MARI = a/b$, where a is the number of antibiotics to which isolate was resistant, and b is the total number of antibiotics against which individual isolate was tested.

5.6. Determination of the minimum inhibitory concentration (MIC)

Tetracycline and nalidixic acid in powdered form were purchased and stored at 4 ºC until use. The stock solution of each antibiotics was prepared and dilutions were made as described by the CLSI [6]. Minimum inhibitory concentration (MIC) was determined against the resistant isolates using standard micro-broth dilution method conforming to the recommended standards of CLSI [6]. The concentration of tetracycline and nalidixic acid used ranged from 4 µg/ml to 2048 µg/ml. Two-fold serial dilution of the two antibiotics understudy was prepared from the stock solution, after which three to five colonies from overnight pure culture were suspended in sterile normal saline. The turbidity of the suspension was further adjusted to 0.5 McFarland standard. The MIC methodology is described in detailed by Edziri *et al*. [10] with little modification. The lowest concentration of antibiotics without bacteria growth was taken as the MIC value.

5.7. Minimum bactericidal concentration (MBC)

Minimum bactericidal concentration was carried out in accordance to the description of Sudjana *et al*. [40]. A small volume of about 30–40 µl from each microtitre well that showed no visible growth after 24 h of incubation was spread on an already prepared sterile nutrient agar plate that contains no antimicrobial agent using a sterile glass spreader. This assay was carried out in duplicate, each plate was incubated for 24 h at 37 ºC. The lowest concentration of antibiotics that did not produce any growth after incubation was taken as the MBC value.

Abbreviations

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; MARI: multiple antibiotic resistance index; MARPs: multiple antibiotic resistance phenotypes; CLSI: clinical and laboratory standard institute; PCR: polymerase chain reaction.

Declarations

Ethics approval and Consent to participate

Not applicable.

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Author's contributions

O.V.A. performed the experiment, analysed the data, designed and coordinated the manuscript. A.I.O. supervised, reviewed and polished the manuscript. All authors revised and approved the final manuscript.

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Availability of data and materials

Not applicable

Consent for publication

Not applicable

Competing interest

The authors declare no competing interest

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Figures

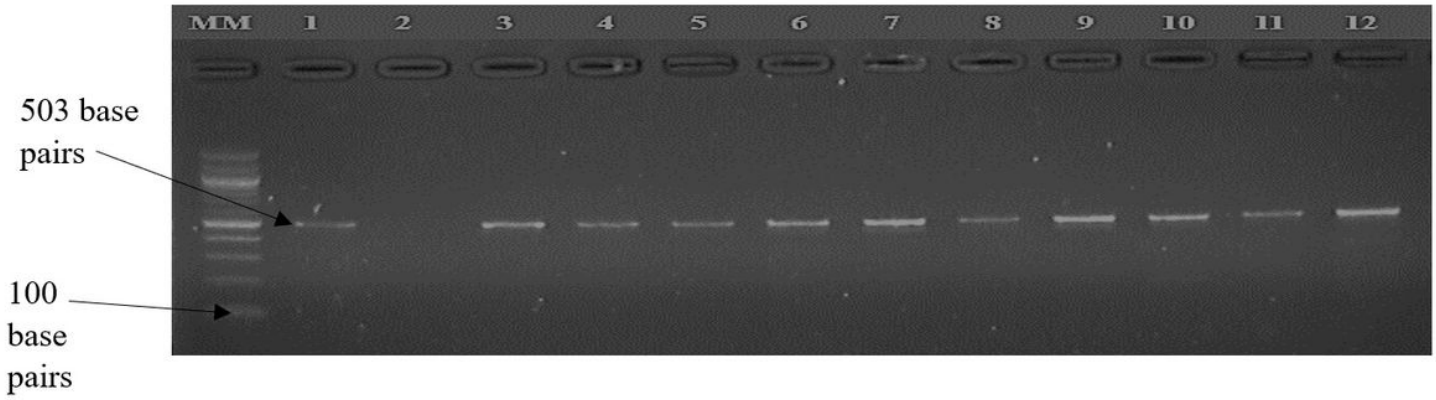

Figure 1

A gel electrophoresis picture showing the confirmation of Vibrio parahaemolyticus using toxR gene. Lane MM: 100 base pair gene marker; lane 1: Positive control (DSM 10027); lane 2: Negative control; lane 3 to 12: Positive isolates.

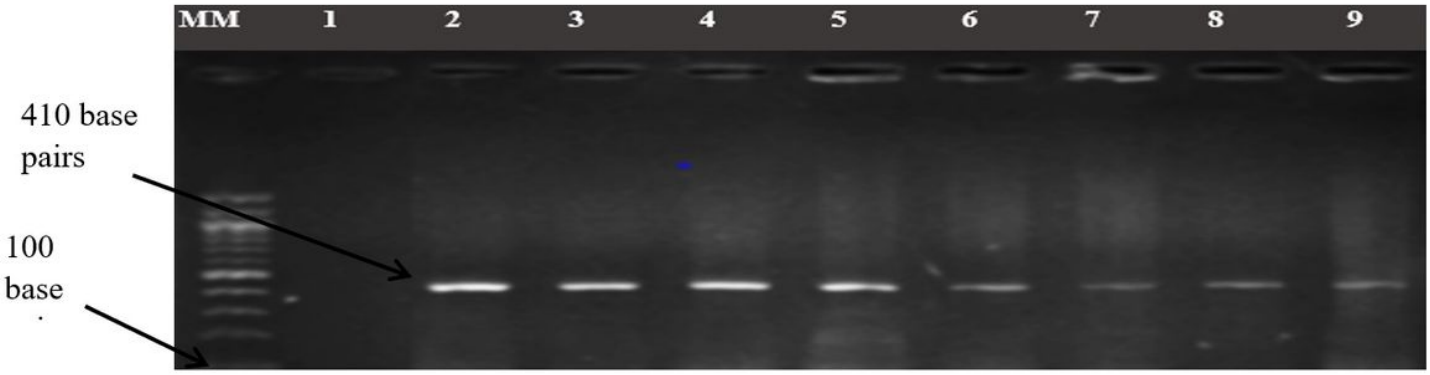

Figure 2

A gel electrophoresis picture showing the confirmation of Vibrio vulnificus using hsp60 gene. Lane MM: 100 base pair gene marker; lane 1: Negative control; lane 2: Positive control (DSM 10143); lane 3 to 9: Positive isolates.

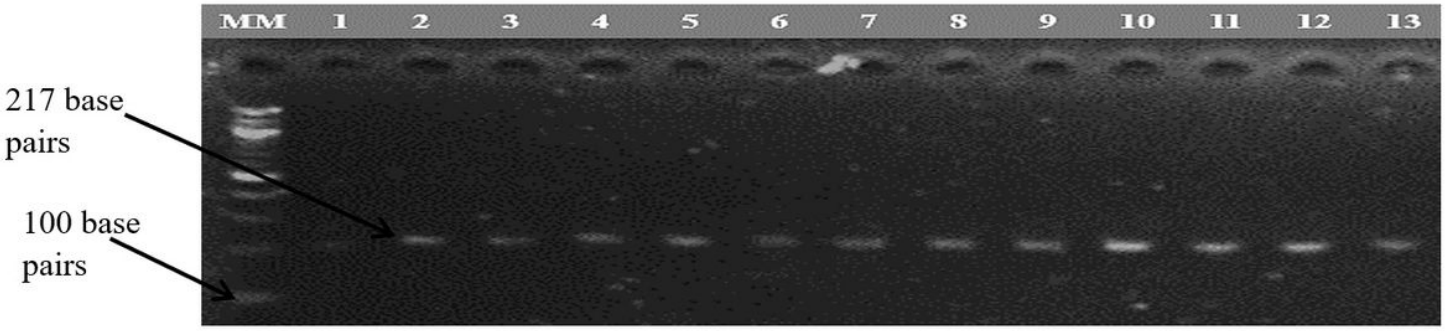

Figure 3

A gel electrophoresis picture showing the confirmation of Vibrio fluvialis using toxR gene. Lane MM: 100 base pair gene marker; lane 1: Negative control; lane 2: positive control (DSM 19283); lane 3 to 13: Positive isolates.