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ISID Research Grant Report

Multi-drug resistant Vibrio species with putative invasive and toxigenic signatures isolated from abattoir effluents in the Niger Delta region of Nigeria

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Introduction

An abattoir is a special facility designed and licensed for receiving, holding, slaughtering and inspecting meat animals and meat products before release to the public (Aarestrup 1995). Abattoir inspection of live animals (ante–mortem) and carcass (post–mortem) are critical to surveillance for animal diseases and zoonoses (Nwanta et al. 2008). Cadmus et al. (1999) reported that pathogens of zoonotic importance are associated with more than 80% of public abattoirs in Nigeria. This observation has serious public health implication as many Nigerian abattoirs dispose their effluents directly into streams and rivers without any form of treatment (Alonge 2005). Incidentally, these streams and rivers also serve as water resource for domestic, agricultural, recreational as well as drinking purposes for communities and settlements downstream. It is little wonder therefore that waterborne diseases such as cholera and others are recurring indices in Nigeria.

Reports in the literature (Atieno et al. 2013; Ogbonna 2014) suggests that abattoir effluents were important environmental reservoirs for *Vibrio* species. And given the proposition that environmental reservoir of toxigenic *Vibrio* species and/or non-enterotoxigenic environmental *Vibrio* strains may serve as progenitors for future enterotoxin producing epidemic strains (Colwell and Hug 1994), it becomes imperative to monitor abattoir effluents for potential *Vibrio* pathogens. Moreover, antibiotics are often employed as feed additives to promote rapid growth of livestock (Kümmerer 2003); thereby contributing to increased incidence of antibiotic resistance among bacterial species that inhabit abattoir effluents, due to selective pressure (Aarestrup 1995). Emergence of microbial resistance to multiple drugs is an ongoing challenge that threatens the effectiveness of antibiotics in the continuous management of infectious diseases; especially in low and medium income countries (many of which are in Africa) lacking relevant infrastructures and institutions targeted at making sanitation and water resources available and accessible to all. A good example is a report from Guinea Bissau, stating that multiple antibiotics resistance was responsible for the increase in fatality from 1% to 5.3% during a cholera outbreak that occurred between 1996 and 1997 (Dalsgaard et al. 2000).

Although abattoir effluents have been reported (Atieno et al. 2013; Ogbonna 2014) to be important environmental reservoirs for *Vibrio* species, no study (to the best of our knowledge) has previously evaluated the antibiotic susceptibility patterns of *Vibrio* species with invasive/toxigenic potentials isolated from abattoir effluents in Nigeria. The aim of this study therefore, was to investigate the antibiogram of potentially invasive/toxigenic *Vibrio* species isolated from abattoir effluents in the Niger Delta region of Nigeria.

Materials and Methods

Sample collection and study site

Abattoir effluent was collected from three abattoirs located in the Niger Delta region of Nigeria: Oghara, Delta State (coordinates: 5o55'52.35" N, 5o39'39.86" E); Sapele, Delta State (coordinates: 5o52'34.44" N, 5o41'36.81" E); and Ikpoba hill, Benin City, Edo State

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(coordinates: 6o21'00.67" N, 5o38'34.92" E). Effluent samples were collected aseptically into sterile 1000 ml Nalgene bottles and transported in a cooler box containing ice packs to the laboratory for analyses. Samples were processed within 24 h of collection; in the event of slight delay, samples were refrigerated overnight at 4°C prior to analyses.

Isolation and preliminary identification of Vibrio species

Aliquots of the samples were inoculated into alkaline peptone water (APW, Pronadisa) and incubated aerobically at 37 oC for 18 - 24 h. Turbid cultures were streaked onto thiosulphate citrate bile salts sucrose (TCBS) agar (Pronadisa) and incubated at 37 oC for 24 h. Suspected *Vibrio* species appear as green or yellow colonies on TCBS. Five to ten isolated colonies per plate were randomly picked from each sample and sub-cultured onto fresh TCBS agar plates. The pure isolates were subjected to preliminary identification using standard cultural and biochemical methods as described by Kaysner and DePaola (2004). The identity of presumptive *Vibrio* isolates were further confirmed using PCR technique as described below.

Molecular confirmation of Vibrio isolates:

Isolates identified as *Vibrio* species by cultural/biochemical techniques were confirmed by PCR using the specific primers described in Table 1. DNA extraction and PCR were carried out as described by Igbinosa et al. (2009) with slight modifications. Single colonies of presumptive *Vibrio* strains grown overnight at 37 oC on TSA-2% NaCl agar plates were picked, suspended in 200 µl of sterile Milli-Q PCR grade water (Merck SA) and the cells lysed using Dri-block DB.2A (Techne SA) for 15 min at 100 oC. The cell debris were removed by centrifugation at 11, 000 × g for 2 min using a MiniSpin micro centrifuge (Merck SA). The cell lysates (10 µl) were used as DNA template in the PCR assays immediately after extraction or following storage at -20 oC. Sterile Milli-Q PCR grade water (Inqaba Biotec SA) was included in each PCR assay as negative control. The thermal cycling condition was as follows: initial denaturation at 93 oC for 15 min., followed by 35 cycles of denaturation at 92 oC for 40 s, annealing at 57 oC for 1 min and extension at 72 oC for 1.5 min; and a final extension step of 72 oC for 7 min. The amplified products were held at 4 oC after completion of the cycles prior to electrophoresis. For *V. fluvialis* the amplification condition was: initial denaturation at 94 oC for 5 min, followed by 30 cycles consisting of denaturation at 94 oC for 40 s, annealing at 65 oC for 40 s and extension at 72 oC for 1 min. The PCR products were electrophoresed in 1.5 % agarose gel containing 0.5 mg/l ethidium bromide for 40 min. at 100 V and then visualized using a UV transilluminator.

Detection of cholera toxin gene (ctx):

The extraction of genomic DNA from Vibrio isolates was as described above. The protocol described by Kaysner and DePaola (2004) was used for detection of the *ctx* toxigenic gene in suspected strains of *V. cholerae*. The primer set used was, 5'-TGA AAT AAA GCA GTC AGG TG-3' (forward) and 5'-GGT ATT CTG CAC ACA AAT CAG-3' (reverse); and the size of the expected PCR amplicon is 777 bp. The amplification reaction consisted of an initial denaturation step of 94 oC for 3 min and 35 cycles of 1 min. at 94 oC, 1 min. at 55 oC, and 1 min. at 72 oC; with a final extension step of 3 min. at 72 oC.

Detection of invasive genes:

Genomic DNA extraction from isolates as well as PCR reaction was carried out according to the procedure of Igbinosa et al. (2009) as described above. The primer sets for the reaction are given in Table 1 and include *Fp.flaE. 79F, Vp.flaE-934R* and *Vv.hsp-326F* and *Vv.hsp-697R*.

Antibiotic susceptibility test:

Susceptibility of *Vibrio* isolates to antimicrobial agents was performed by disc diffusion method

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following guidelines established by Clinical and Laboratory Standards Institute (CLSI 2005) and using commercial antimicrobial discs. A total of 19 antibiotic discs (Mast Diagnostics, Merseyside, United Kingdom) commonly used in human therapy were employed in the antibiogram test; they include: ofloxacin (OFX; 50µg), ceftazidime (CAZ; 30µg), cefixime (CXM; 30µg), kanamycin (K; 30 µg), tetracycline (T; 30µg), trimethoprim (TM; 2.5µg), gentamycin (GM; 10µg), rifampicin (RP; 5µg), nalidixic acid (NA; 30µg), amikacin (AK; 30µg), ampicillin (AP; 10µg), amoxicillin (A; 10µg), netilmicin (NET; 10µg), imipemem (IMI; 10µg), streptomycin (S; 10µg), ciprofloxacin (CIP; 5µg), trimethoprim-sulfamatoxazole [(TS;T (1.25µg); S (23.75µg)], chloramphenicol (C; 30µg) and ceftriaxone (CRO; 30µg).

Multiple antibiotic resistances (MAR) index

MAR index was calculated as previously described by Blasco et al. (2008) as follows:

$$MAR = a/b$$

where a = number of antibiotics to which an isolate was resistant;

b = total number of antibiotics against which individual isolates were tested.

MAR index higher than 0.2 identifies organisms that originate from high-risk sources of contamination, where antibiotics are often used or abused (Odjadjare et al. 2012).

Results

Out of a total of 150 presumptive *Vibrio* isolates identified using cultural/biochemical techniques 48 (32%) were confirmed to be *Vibrio* species by PCR analysis (Figures 1 to 6). Twenty three (23(47.9%)) of these isolates were *Vibrio cholerae*; 11(22.9%) *V. fluvialis;* 8(16.7%) *V. vulnificus*; and 6(12.50%) *V. parahaemolyticus*. Twenty one (21) of the confirmed isolates belonging to four species (6 *Vibrio cholerae*; 5 *V. parahaemolyticus*; 7 *V. vulnificus*; and 3 *V. fluvialis*) were randomly selected for the antibiogram assay.

The isolates (except strains of *V. vulnificus*) were generally resistant to ampicillin (60-67%), trimethoprim (80-100%) and tetracycline (60-83%) (Table 2). In addition, strains of *Vibrio cholerae* were resistant to trimethoprim-sulfamethoxazole (83%), cefixime (67%) and rifampicin (67%); while *V. parahaemolyticus* were resistant to amoxicillin (60%); whereas *V. fluvialis* showed resistance to trimethoprim-sulfamethoxazole. *V. vulnificus* were generally sensitive to the test antibiotics, with a few showing low resistance to ceftazidime and trimethoprim among others (Tables 2).

The isolates were generally sensitive to ceftriaxone (86-100%), the aminoglycosides (67-100%), imipenem (86-100%), ofloxacin (83-100%) and chloramphenicol (67-100%). Although majority of the isolates were sensitive to amoxicillin (67-86%), *V. parahaemolyticus* showed low susceptibility (40%) to this antibiotics. The isolates were also sensitive to ceftazidime (60-67%), except *V. vulnificus*, which exhibited reduced sensitivity (29%) to the antibiotics. Similarly, many of the isolates were sensitive to cefixime (60-80%), except *V. cholerae* which showed low sensitivity at 17%. Ciprofloxacin showed good activity against *V. parahaemolyticus* (80%) and *V. vulnificus* (100%), but exhibited reduced sensitivity against *V. cholerae* (50%) and *V. fluvialis* (33%). Whereas, nalidixic acid was active against majority of the isolates (60-100%), it exhibited reduced sensitivity against *V. vulnificus*. Rifampicin was efficacious against majority of the isolates (60-100%), except *V. cholerae* which showed very low (17%) sensitivity to the antibiotic (Table 2).

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Table 3 shows the resistance pattern of the test isolates assessed in this study. From the table it was observed that two strains of *V. vulnificus* were completely susceptible to all the test antibiotics deployed in this study; and hence have no resistance record. The other isolates exhibited multiple antibiotic resistance (MAR) in combinations ranging from 2 to 12 antibiotics except in a strain of *V. parahaemolyticus* and *V. vulnificus* which exhibited mono-resistances to ceftazidime and tetracycline respectively (Table 3). The MAR index ranged between 0 and 0.63; the highest MAR index was observed in a strain of *Vibrio cholerae* isolated from Oghara, while the lowest was expectedly observed in the two strains of *V. vulnificus* which were completely sensitive to all the test antibiotics.

Discussion

The PCR confirmation of 48 out of 150 (32%) presumptive isolates as true *Vibrio* spp. is consistent with the report of Costa et al. (2015), which suggested that phenotypic identification alone, often leads to misidentification of microorganisms. The detection of virulence and invasive gene determinants (*ctx, hsp60* and *flaE* genes) in a number of *Vibrio* isolates in this study, indicates that abattoir effluent is an important repository of pathogenic *Vibrio* species; and could be a considerable contributor to the recurrent episodes of epidemic cholera and other non-*Vibrio cholerae* outbreaks in Nigeria. This observation is in agreement with reports elsewhere (Chakraborty et al. 2000; Mukhopadhyay et al. 2001), which suggests that demonstration of the existence of environmental strains of *Vibrio* spp. which carry one or more virulence genes or their homologues supports the possibility of an environmental origin for pathogenic vibrios.

Consistent with the observation of this study, Igbinosa et al. (2009), reported considerable resistance of *Vibrio* isolates from municipal wastewater against ampicillin, trimethoprim, and trimethoprim/sulphamethoxazole in South Africa; while Marin et al. (2013) documented resistance against trimethoprim and trimethoprim/sulphamethoxazole amongst clinical *Vibrio* strains isolated from different parts of Nigeria. The observation of resistance against trimethoprim is worrisome, as the antibiotic was previously reported to be the drug of choice for the treatment of cholera in children and pregnant women (Thungapathra et al. 2002). Strains of *Vibrio* tested in this study (except V. vulnificus) were generally resistant to tetracycline (60 – 83%), in agreement with reports from Tanzania and Rwanda, but contrary to reports from Kenya, South Sudan, South Africa, Somalia (Materu et al. 1997) and Northern Nigeria (Opajobi et al. 2004).

Isolates of the current study exhibited remarkable sensitivity to ceftriaxone and imipenem (Table 2), in agreement with the report of Chiang and Chuang (2003) who observed that imipenem and the cephalosporins, including ceftriaxone were effective against *Vibrio* infections. However, contrary to the submission of Chiang and Chuang (2003), *V. vulnificus* and *Vibrio cholerae* in this study exhibited reduced sensitivity and resistance to ceftazidime (29%) and cefixime (17%) respectively (Table 2). Li et al. (2003) reported remarkable sensitivity to the aminoglycosides (streptomycin and kanamycin) in agreement with the observation of this study; however, reports elsewhere (Ottaviani et al. 2001; Marin et al. 2013) suggested otherwise. *Vibrio* strains in this study were also considerably sensitive to nalidixic acid, ofloxacin, ciprofloxacin, chloramphenicol and rifampicin (Table 2), contrary to the observation of Ottaviani et al. (2001), who reported resistance against rifampicin. Marin et al. (2013) also reported an intermediate/reduced sensitivity to chloramphenicol and ciprofloxacin, and resistance against nalidixic acid, contrary to the observation of this study. However, consistent with the observation of this study Li et al. (1999) reported sensitivity of *Vibrio* strains isolated

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from cultured silver sea bream to rifampicin; while Opajobi et al. (2004) observed sensitivity of epidemic strains of *Vibrio cholerae* to ofloxacin.

Seventeen (17) out of the 21 (81 %) isolates tested for antibiogram exhibited multiple antibiotic resistances (MAR) ranging from two to twelve antibiotics with distribution across 10 classes of antibiotics. Consistent with the observation of this study Igbinosa et al. (2009) reported MAR patterns ranging from 5 – 10 antibiotics. However, the percentage of isolates exhibiting MAR as reported by Igbinosa and coworkers (10-20%) were relatively lower than those (81%) observed in this study. The MAR indices observed in this study were higher than the 0.2 limit in 14 (67%) of the test isolates (Table 3); indicating that many of the isolates originated from high risk sources of contamination where antibiotics were often used or abused (Odjadjare et al. 2012). Abattoir effluents are considered to be one of such high risk sources of contamination since it is associated with waste from livestock which are often bred by feeds containing antibiotics additives. The residual antibiotics that enters the environment with abattoir waste effluent have been reported (Aarestrup 1995; Kümmerer 2003) to exert selective pressure on microbial populations contained therein, thereby enhancing MAR as observed in this study.

The current study demonstrated that abattoir effluents are important reservoirs of multidrug resistant Vibrio pathogens with invasive/toxigenic potentials. This implies that abattoir effluents could be important contributors to the recurrent episodes of epidemic cholera and non-Vibrio cholerae outbreaks in Nigeria. We therefore recommend a thorough surveillance initiative by relevant stakeholders to elucidate the extent to which abattoir effluents contribute to the spread and recurrence of epidemic vibriosis in Nigeria (and possibly elsewhere) with a view to arresting the scourge of vibriosis (including cholera) in our society.

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Table 1: List of primers used in this study.

Target species	Primer	Sequences (5'- 3')	Target gene	Amplicon size (bp)
All *Vibrio* spp	*V.* 16S-700F	CGG TGA AAT GCG TAG AGA T	16S rRNA	663
	V. 16S-1325R	TTA CTA GCG ATT CCG AGT TC		
V. cholera	*Vc. sodB*-F	AAG ACC TCA ACT GGC GGT A	*sodB*	248
	Vc. sodB-R	GAA GTG TTA GTG ATC GCC AGA GT		
V. parahaemolyticus	*Vp. flaE*-79F	GCA GCT GAT CAA AAC GTT GAG T	*flaE*	897
	Vp. flaE-934R	ATT ATC GAT CGT GCC ACT CAC		
V. vulnificus	*Vv. hsp*-326F	GTC TTA AAG CGG TTG CTG C	*hsp60*	410
	Vv. hsp-697R	CGC TTC AAG TGC TGG TAG AAG		
V. fluvialis	*Vf- toxR* F	GAC CAG GGC TTT GAG GTG GAC GAC	*toxR*	217
	Vf- toxR R	AGG ATA CGG CAC TTG AGT AAG ACTC		

Table 2. Antibiotics susceptibility profile of the *Vibrio* strains isolated from abattoir effluents

ANTIBIOTICS CLASS	ANTIBIOTICS	Percentage (%) response of isolates to antibiotics											
		Vibrio cholera (n=6)			*V. parahaemolyticus* (n=5)			*V. vulnificus* (n=7)			*V. fluvialis* (n=3)		
		S	I	R	S	I	R	S	I	R	S	I	R
Penicillins	Ampicillin	17	0	83	40	0	60	71	0	29	0	33	67
	Amoxicillin	67	0	33	40	0	60	86	0	14	67	0	33
Cephems	Ceftazidime	67	0	33	60	0	40	29	29	43	67	0	33
	Cefixime	17	17	67	60	20	20	86	14	0	67	33	0
	Cefriaxone	100	0	0	100	0	0	86	0	14	100	0	0
Aminoglycosides	Gentamycin	67	0	33	100	0	0	100	0	0	100	0	0
	Amikacin	100	0	0	100	0	0	100	0	0	100	0	0
	Kanamycin	100	0	0	100	0	0	100	0	0	100	0	0
	Netilmicin	83	0	17	100	0	0	100	0	0	100	0	0
	Streptomycin	67	0	33	100	0	0	86	0	14	67	33	0
Folate Pathway Inhibitor	Trimethoprim	0	0	100	20	0	80	57	0	43	0	0	100
	Trimet/sulpha.[a]	17	0	83	60	0	40	57	14	29	33	0	67
Fluoroquinolones	Ofloxacin	83	0	17	100	0	0	100	0	0	100	0	0
	Ciprofloxacin	50	17	33	80	20	0	100	0	0	33	33	33
Quinolones	Nalidixic acid	100	0	0	60	0	40	43	29	29	67	33	0
Tetracyclines	Tetracycline	17	0	83	40	0	60	86	0	14	33	0	67
Carbapenems	Imipemem	100	0	0	100	0	0	86	0	14	100	0	0
Phenicol	Chloramphenicol	67	17	17	100	0	0	86	0	14	100	0	0
Ansamycins	Rifampicin	17	17	67	60	20	20	86	14	0	100	0	0

Legend: [a] Trimethoprim/sulphamethoxazole S- Sensitive I- Intermediate R- resistant

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Table 3. Multiple antibiotic resistance index of the *Vibrio* isolates

ISOLATE CODE	NAME OF SPECIES	ANTIBIOTIC RESISTANCE PATTERN	MAR INDEX
[a]O_7	*Vibrio cholerae*	TM, TS, AP, T, A, CIP,	0.32
[b]I_2	*Vibrio cholerae*	TM, AP, T, RP,	0.21
[a]O_5	*Vibrio cholerae*	CXM, TM, TS, AP, T, RP	0.32
[a]DPA Y1	*Vibrio cholerae*	T, TS, RP, CAZ, CXM,GM, A, CIP, TM, NET, AP, S	0.63
[b]I_1	*Vibrio cholerae*	TS, CAZ, CXM, C, GM, TM, OFX	0.37
[a]O_3	*Vibrio cholerae*	T, TS, RP, CXM, TM, AP, S	0.37
[a]O_2	*V. parahaemolyticus*	TM, TS, AP, T, A	0.26
[b]I_4	*V. parahaemolyticus*	CXM, TM, TS, AP, T, A	0.32
[b]I_5	*V. parahaemolyticus*	CAZ, TM, AP, T, NA, RP, A	0.37
[a]O_8	*V. parahaemolyticus*	TM, NA	0.11
[a]O_4	*V. parahaemolyticus*	CAZ,	0.05
[c]S_1	*Vibrio vulnificus*	NA, A, IMI, S, C	0.26
[b]I_7	*Vibrio vulnificus*	T	0.05
[c]S_2	*Vibrio vulnificus*	CAZ, TM, CRO,	0.16
[c]S_3	*Vibrio vulnificus*	NIL	0.00
[b]TS_{30}	*Vibrio vulnificus*	NIL	0.00
[b]TS_{70}	*Vibrio vulnificus*	TS, CAZ, TM, AP	0.21
[b]TS_{72}	*Vibrio vulnificus*	TS, NA, TM, AP	0.21
[a]O_6	*Vibrio fluvialis*	TM, TS, AP, T,	0.21
[c]S_4	*Vibrio fluvialis*	CAZ, TM	0.11
[b]I_8	*Vibrio fluvialis*	T, TS, A, CIP, TM, AP	0.32

Legend: OFX, ofloxacin; CAZ, ceftazidime; CXM, cefixime, K, kanamycin; T, tetracycline; TM, trimethoprim; GM, gentamycin; RP, rifampicin; NA, nalidixic acid; AK, amikacin; AP, ampicillin; A, amoxicillin; NET, netilmicin; IMI, imipemem; S, streptomycin; CIP, ciprofloxacin; TS, trimethoprim-sulfametoxazole; C, chloramphenicol; and CRO, ceftriaxone.

[a] Isolates from Oghara abattoir;

[b] Isolates from Ikpoba abattoir;

[c] Isolates from Sapele abattoir.

ISID Report of Emmanuel E.O. Odjadjare

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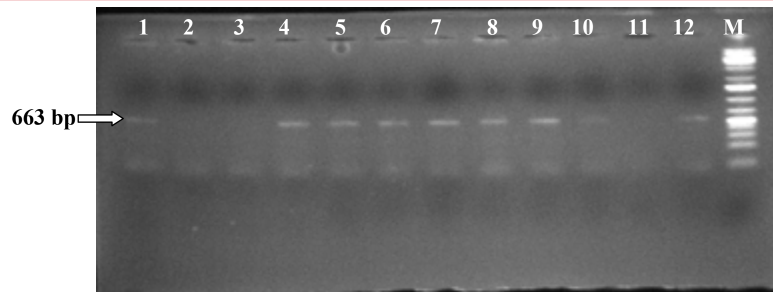

Fig. 1 PCR products of amplified Vibrio genus V.16S – 700 gene

Lanes 1, 4 – 9 and 12 positive samples; Lanes 2 and 3, negative samples; Lane 11 negative control; Lane M 100 bp DNA ladder.

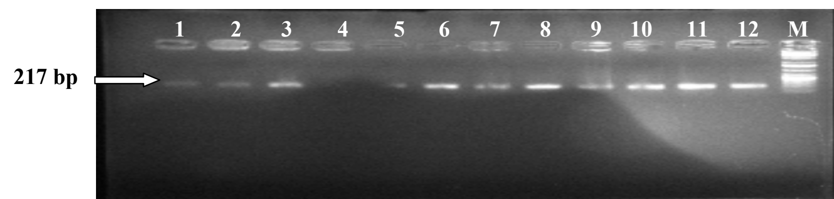

Fig. 2. PCR products of amplified *Vibrio fluvialis* V-*toxR* gene.

Lanes 1, 2, 3, 5-12 positive sample; Lane 4 negative control; Lane M 100 bp DNA ladder

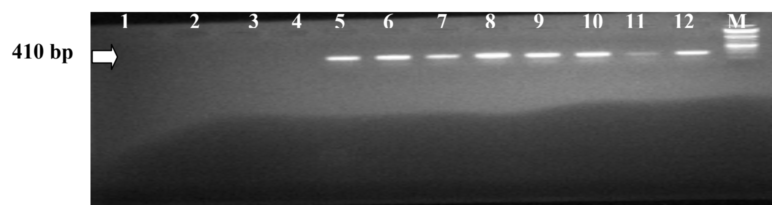

Fig. 3. PCR product of amplified *Vibrio vulnificus* invasive gene (V-*hsp60*)

Lane 1 negative control; Lanes 2- 4 negative samples; Lanes 5 -12 positive sample; Lane M, 100 bp DNA ladder

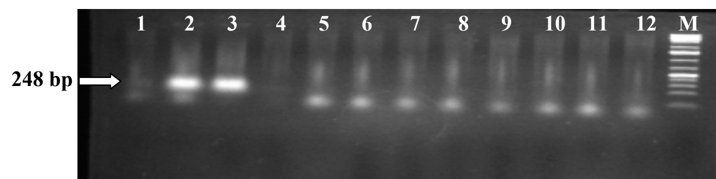

Fig. 4. PCR products of amplified *Vibrio cholerae* V-*sob* gene

Lanes 1, 4-12, negative samples; Lanes 2 and 3 positive samples; Lane M- 100 bp DNA ladder..

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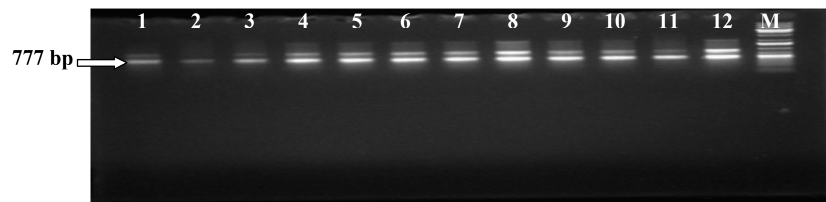

Fig. 5. PCR product of amplified *Vibrio cholerae* enterotoxin gene (V-*ctx*)

Lanes 1-12, positive samples; Lane M, 100 bp DNA ladder.

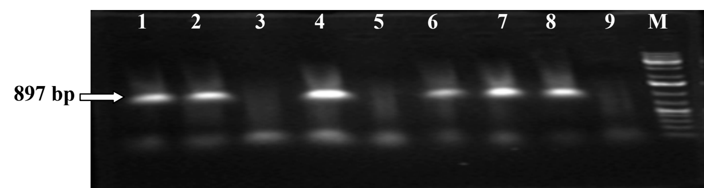

Fig. 6. PCR product of amplified *Vibrio parahaemolyticus* invasive gene (V-*flaE*)

Lanes 1,2,4, 6,7, 8, positive samples; Lanes 3 and 5, negative samples; Lane 9, negative control; Lane M, 100 bp DNA ladder.