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*Original Article*

# Multidrug-resistant *Vibrio* species isolated from abattoir effluents in Nigeria

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## Abstract
Introduction: The antibiograms of *Vibrio* species isolated from abattoir effluents in the Niger Delta region of Nigeria were investigated with respect to their public health significance.
Methodology: *Vibrio* species were isolated and identified using standard microbiological and molecular techniques, while antibiogram of isolates was tested and interpreted using the disk diffusion method described by the Clinical and Laboratory Standards Institute.
Results: Of 150 presumptive isolates, 48 (32%) were confirmed to be *Vibrio* spp. by PCR; of these, 23 (47.9%) were *V. cholerae*, 11 (22.9%) were *V. fluvialis*, 8 (16.7%) were *V. vulnificus*, and 6 (12.5%) were *V. parahaemolyticus*. The antibiogram revealed that *Vibrio* species were generally resistant to ampicillin (60%–67%), trimethoprim (80%–100%), and tetracycline (60%–83%), whereas they were sensitive to ceftriaxone (86%–100%), the aminoglycosides (67%–100%), imipenem (86%–100%), ofloxacin (83%–100%), and chloramphenicol (67%–100%). The isolates exhibited multiple antibiotic resistance (MAR) with an average MAR index of 0.23.
Conclusions: This study demonstrated that abattoir effluents are important reservoirs for multidrug-resistant *Vibrio* species that might be considerable contributors to the recurrent episodes of epidemic cholera and non-*Vibrio* cholera infections in Nigeria.

**Key words:** *Vibrio;* multidrug resistance; abattoir effluent.

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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 [1]. Abattoir inspection of live animals (ante-mortem) and carcasses (post-mortem) is critical to surveillance for animal diseases and zoonoses [2]. Cadmus *et al*. [3] reported that pathogens of zoonotic importance are associated with more than 80% of public abattoirs in Nigeria. This observation has serious public health implications, as many Nigerian abattoirs dispose their effluents directly into streams and rivers without any form of treatment [4]. Incidentally, these streams and rivers also serve as water resources 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 are recurring in Nigeria.

Antibiotics are often employed as feed additives to promote rapid growth of livestock [5], thereby contributing to increased incidence of antibiotic resistance among bacterial species that inhabit abattoir effluents, due to selective pressure [1]. 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 infrastructure and institutions targeted at making sanitation and water resources available and accessible to all. A good example is a work from Guinea Bissau that reported that multiple-antibiotic resistance was responsible for the increase in fatality from 1% to 5.3% during a cholera outbreak that occurred between 1996 and 1997 [6].

Although abattoir effluents have been reported [7,8] to be important environmental reservoirs for *Vibrio* species, there is a dearth of information in the literature on antibiotic susceptibility patterns of *Vibrio* species isolated from abattoir effluents in Nigeria. Therefore, the aim of this study was to investigate the antibiogram of *Vibrio* species isolated from abattoir effluents in the Niger Delta region of Nigeria.

## Methodology

*Sample collection and study site*

Abattoir effluent was collected from three abattoirs located in the Niger Delta region of Nigeria: Oghara (coordinates: 5°55'52.35" N, 5°39'39.86" E), Delta State; Sapele (coordinates: 5°°52'34.44" N, 5°41'36.81" E), Delta State; and Ikpoba Hill (coordinates: 6°21'00.67" N, 5°38'34.92" E), Benin City, Edo State. Effluent samples were collected aseptically into sterile 1,000 mL Nalgene bottles and transported in a cooler box containing ice packs to the laboratory for analyses. Samples were processed within 24 hours 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, Madrid, Spain) and incubated aerobically at 37°C for 18 to 24 hours. Turbid cultures were streaked onto thiosulphate citrate bile salts sucrose (TCBS) agar (Pronadisa, Madrid, Spain) and incubated at 37°C for 24 hours. 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 [9]. The identity of presumptive *Vibrio* isolates were further confirmed using the polymerase chain reaction (PCR) technique 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.* [10], with slight modifications. Single colonies of presumptive *Vibrio* strains grown overnight at 37°C on TSA-2% NaCl agar plates were picked, suspended in 200 µL of sterile Milli-Q PCR grade water (Merck, Modderfontein, South Africa), and the cells lysed using Dri-block DB.2A (Techne, Cape Town, South Africa) for 15 minutes at 100°C. The cell debris were removed by centrifugation at 11,000 × g for 2 minutes using a MiniSpin micro centrifuge (Merck, Modderfontein, South Africa). The cell lysates (10 µL) were used as a DNA template in the PCR assays immediately after extraction or following storage at -20°C. Sterile Milli-Q PCR-grade water (Inqaba Biotec, Pretoria, South Africa) was included in each PCR assay as a negative control. The thermal cycling condition was as follows: initial denaturation at 93°C for 15 minutes, followed by 35 cycles of denaturation at 92°C for 40 seconds, annealing at 57°C for 1 minute and extension at 72°C for 1.5 minute, and a final extension step of 72°C for 7 minutes. The amplified products were held at 4°C after completion of the cycles prior to electrophoresis. For *V. fluvialis*, the amplification condition was as follows: initial denaturation at 94°C for 5 minutes, followed by 30 cycles consisting of denaturation at 94°C for 40 seconds, annealing at 65°C for 40 seconds, and extension at 72°C for 1 minute. The PCR products were electrophoresed in 1.5% agarose gel containing 0.5 mg/L ethidium bromide for 40 minutes 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 [9] 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

**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<br>*V.* 16S-1325R | CGG TGA AAT GCG TAG AGA T<br>TTA CTA GCG ATT CCG AGT TC | 16S rRNA | 663 |
| *V. cholera* | *Vc. sodB*-F<br>*Vc. sodB*-R | AAG ACC TCA ACT GGC GGT A<br>GAA GTG TTA GTG ATC GCC AGA GT | *sodB* | 248 |
| *V. parahaemolyticus* | *Vp. flaE*-79F<br>*Vp. flaE*-934R | GCA GCT GAT CAA AAC GTT GAG T<br>ATT ATC GAT CGT GCC ACT CAC | *flaE* | 897 |
| *V. vulnificus* | *Vv. hsp*-326F<br>*Vv. hsp*-697R | GTC TTA AAG CGG TTG CTG C<br>CGC TTC AAG TGC TGG TAG AAG | *hsp60* | 410 |
| *V. fluvialis* | *Vf- toxR* F<br>*Vf- toxR* R | GAC CAG GGC TTT GAG GTG GAC GAC<br>AGG ATA CGG CAC TTG AGT AAG ACTC | *toxR* | 217 |

Source: Igbinosa et al. [11]

the expected PCR amplicon was 777 bp. The amplification reaction consisted of an initial denaturation step of 94°C for 3 minutes and 35 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C, with a final extension step of 3 minutes at 72°C.

*Antibiotic susceptibility test*

Susceptibility of *Vibrio* isolates to antimicrobial agents was performed using the disk diffusion method following guidelines established by the Clinical and Laboratory Standards Institute [11] and using commercial antimicrobial disks. A total of 19 antibiotic disks (Mast Diagnostics, Merseyside, United Kingdom) commonly used in human therapy were employed in the antibiogram test; they included ofloxacin (OFX; 50 µg), ceftazidime (CAZ; 30 µg), cefixime (CXM; 30 µg), kanamycin (K; 30 µg), tetracycline (T; 30 µg), trimethoprim (TM; 2.5 µg), gentamycin (GM; 10 µg), rifampicin (RP; 5 µg), nalidixic acid (NA; 30 µg), amikacin (AK; 30 µg), ampicillin (AP; 10 µg), amoxicillin (A; 10 µg), netilmicin (NET; 10 µg), imipemem (IMI; 10 µg), streptomycin (S; 10 µg), ciprofloxacin (CIP; 5 µg), trimethoprim-sulfamatoxazole (TS; T [1.25 µg], S [23.75 µg]), chloramphenicol (C; 30 µg), and ceftriaxone (CRO; 30 µg).

*Multiple antibiotic resistance (MAR) index*

MAR index was calculated as previously described by Blasco *et al*. [12] as MAR = a/b, where a is the number of antibiotics to which an isolate was resistant and b is the 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 [13].

## 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. Of these isolates, 23 (47.9%) were *V. cholerae*; 11 (22.9%) were *V. fluvialis*, 8 (16.7%) were *V. vulnificus*, and 6 (12.50%) were *V. parahaemolyticus*. A total of21 of the confirmed isolates belonging to four species (6 *V. 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 *V. cholerae* were resistant to trimethoprim-sulfamethoxazole (83%), cefixime (67%), and rifampicin (67%), while *V. parahaemolyticus* were resistant to amoxicillin (60%), and *V. fluvialis* showed resistance to trimethoprim-

**Table 2.** Antibiotic susceptibility profiles 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 |
| | Ceftriaxone | 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 |
| | T/* | 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 | Imipenem | 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 |

* Trimethoprim/sulphamethoxazole; S: sensitive; I: intermediate; R: resistant.

sulfamethoxazole. *V. vulnificus* were generally sensitive to the test antibiotics, with a few showing low resistance to ceftazidime and trimethoprim, among others (Table 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 the majority of the isolates were sensitive to amoxicillin (67%–86%), *V. parahaemolyticus* showed low susceptibility (40%) to this antibiotic. The isolates were also sensitive to ceftazidime (60%–67%), except for *V. vulnificus*, which exhibited reduced sensitivity (29%) to the antibiotic. Similarly, many of the isolates were sensitive to cefixime (60%–80%), except for *V. cholerae*, which showed low sensitivity (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%). Nalidixic acid was active against the majority of the isolates (60%–100%), but it exhibited reduced sensitivity against *V. vulnificus*. Rifampicin was effective against the majority of the isolates (60%–100%), except for *V. cholerae*, which showed very low (17%) sensitivity to the antibiotic (Table 2).

Table 3 shows the resistance pattern of the test isolates assessed in this study. Two strains of *V. vulnificus* were completely susceptible to all the test antibiotics deployed in this study and hence had no resistance record. The other isolates exhibited MAR in combinations ranging from 2 to 12 antibiotics, except in strains of *V. parahaemolyticus* and *V. vulnificus* that exhibited mono-resistance 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 *V. cholerae* isolated from Oghara, while the lowest was expectedly observed in the two strains of *V. vulnificus* that were completely sensitive to all the tested antibiotics.

## Discussion

The PCR confirmation of 48 of 150 (32%) presumptive isolates as true *Vibrio* spp. is consistent with the report of Costa *et al.* [14], which suggested that phenotypic identification alone often leads to misidentification of microorganisms and/or wrong diagnosis of infections. Knowledge of the true identity of potential bacterial pathogens is more often than not critical to effective treatment, management, and control of bacterial infections [15]. Hence, increased attention is being focused on nucleic acid-based characterization of bacterial isolates due to the relative sensitivity and specificity of such techniques.

**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 |

[a] Isolates from Oghara abattoir; [b] Isolates from Ikpoba abattoir; [c] Isolates from Sapele abattoir; MAR: multiple antibiotic resistance; 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: imipenem; S: streptomycin; CIP: ciprofloxacin; TS: trimethoprim-sulfamethoxazole; C: chloramphenicol; CRO: ceftriaxone.

Consistent with the observation of this study, Igbinosa *et al.* [10] reported considerable resistance of *Vibrio* isolates from municipal wastewater against ampicillin, trimethoprim, and trimethoprim/sulphamethoxazole in South Africa, while Marin *et al*. [16] documented resistance against trimethoprim and trimethoprim/sulphamethoxazole among 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 [17]. 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 [18], and northern Nigeria [19].

Isolates of the current study exhibited remarkable sensitivity to ceftriaxone and imipenem (Table 2), in agreement with the report of Chiang and Chuang [20], who observed that imipenem and the cephalosporins, including ceftriaxone, were effective against *Vibrio* infections. However, contrary to the report of Chiang and Chuang [20], *V. vulnificus* and *V. cholerae* in this study exhibited reduced sensitivity and resistance to ceftazidime (29%) and cefixime (17%), respectively (Table 2). Li *et al*. [21] reported remarkable sensitivity to the aminoglycosides (streptomycin and kanamycin), in agreement with the observation of this study; however, other reports [16,21] 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*. [22], who reported resistance against rifampicin. Marin *et al.* [16] 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*. [21] reported sensitivity of *Vibrio* strains isolated from cultured silver sea bream to rifampicin, while Opajobi *et al.* [19] observed sensitivity of epidemic strains of *V. cholerae* to ofloxacin.

Of the 21 (81%) isolates tested for antibiogram, 17MAR, ranging from 2 to 12 antibiotics with distribution across 10 classes of antibiotics. Consistent with the observation of this study, Igbinosa *et al*. [10] reported MAR patterns ranging from 5–10 antibiotics. However, the percentage of isolates exhibiting MAR as reported by Igbinosa *et al*. (10%–20%) was relatively lower than that (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 [13]. Abattoir effluents are considered to be one of such high-risk sources of contamination since they are associated with waste from livestock, which are often fed feed containing antibiotic additives. Residual antibiotics that enter the environment with abattoir waste effluent have been reported [1,5] to exert selective pressure on microbial populations contained therein, thereby enhancing MAR, as observed in this study.

## Conclusions

The current study demonstrated that abattoir effluents are important reservoirs of multidrug-resistant *Vibrio* species that could be considerable contributors to the recurrent episodes of epidemic cholera and non-*Vibrio* cholera 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 ending the scourge of vibriosis (including cholera) in our society.

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