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Bacterial predatory potentials of indigenous *Bdellovibrio* species in southwest Nigeria: Targeting multidrug-resistant *Klebsiella pneumoniae* and *Enterobacter* species isolated from chronic wound infections

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Abstract:

Background: Chronic wound infections could become life-threatening conditions when caused by multi-drug-resistant Gram-negative ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp) pathogens. Alternative therapeutic approaches are vital for the therapy of infections caused by these pathogens and one of the biocontrol therapies is the application of *Bdellovibrio* spp, a Gram-negative bacterium with predatory potential against pathogenic Gram-negative bacteria.
Methodology: This study isolated, and phenotypically and genotypically characterized *Bdellovibrio* spp from environmental sources including soil, pond waters, and faecal materials from cattle and poultry in Osogbo, southwest Nigeria using double-layered agar plating technique, with *Klebsiella* spp as prey. The prey range and predatory potentials of the isolated *Bdellovibrio* spp against 25 clinical bacterial isolates including 6 *K. pneumoniae* and 19 *Enterobacter* spp, recovered from chronic wound infections, was assessed using the same double-layered agar technique.
Results: Five strains of *Bdellovibrio* isolated formed characteristic plaques on lawns of *Klebsiella* spp used as prey. At the molecular level, all the five strains, designated UAB, STU, PW2, PW5, and PW6, belonged to the genus *Bdellovibrio*, however, they exhibited different clustering patterns on the phylogenetic tree. Additionally, the prey range of the five *Bdellovibrio* strains on the bacterial isolates varied, with UAB, PW6, PW5, and PW2 preying upon 12 (48.0%), 12 (48.0%), 7 (28.0%), and 1 (4.0%) bacterial isolate respectively, while STU did not prey on any of the bacteria isolates.
Conclusion: This research presents the first molecular characterization of *Bdellovibrio* spp in Nigeria, hence serving as a baseline study for future *Bdellovibrio* research in Nigeria. It also highlights the promising potential of *Bdellovibrio* spp in control of *K. pneumoniae* and *Enterobacter* spp associated with chronic wound infections.

Keywords: *Bdellovibrio*; multi-drug resistance; *Klebsiella*; *Enterobacter*; wound; predatory bacteria

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Potentiels de prédation bactérienne des espèces indigènes de *Bdellovibrio* dans le sud-ouest du Nigéria: ciblage des espèces de *Klebsiella pneumoniae* et d'*Enterobacter* multirésistantes isolées à partir d'infections de plaies chroniques

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Résumé:

Contexte: Les infections chroniques des plaies peuvent devenir des maladies potentiellement mortelles lorsqu'elles sont causées par des agents pathogènes Gram-négatifs multirésistants ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* et *Enterobacter* spp). Des approches thérapeutiques alternatives sont vitales pour le traitement des infections causées par ces agents pathogènes et l'une des thérapies de lutte biologique est l'application de *Bdellovibrio* spp, une bactérie Gram-négative ayant un potentiel prédateur contre les bactéries Gram-négatives pathogènes.
Méthodologie: Cette étude a isolé et caractérisé phénotypiquement et génotypiquement *Bdellovibrio* spp à partir de sources environnementales, notamment le sol, les eaux d'étang et les matières fécales de bovins et de volailles à Osogbo, dans le sud-ouest du Nigéria, en utilisant la technique de mise en culture sur gélose à double couche, avec *Klebsiella* spp comme proie. La gamme de proies et le potentiel prédateur des *Bdellovibrio* spp isolés contre 25 isolats bactériens cliniques, dont 6 *K. pneumoniae* et 19 *Enterobacter* spp, récupérés d'infections de plaies chroniques, ont été évalués en utilisant la même technique de gélose à double couche.
Résultats: Cinq souches de *Bdellovibrio* isolées ont formé des plaques caractéristiques sur des pelouses de *Klebsiella* spp utilisées comme proies. Au niveau moléculaire, les cinq souches, désignées UAB, STU, PW2, PW5 et PW6, appartenaient au genre *Bdellovibrio*, mais elles présentaient des schémas de regroupement différents sur l'arbre phylogénétique. De plus, la gamme de proies des cinq souches de *Bdellovibrio* sur les isolats bactériens variait, UAB, PW6, PW5 et PW2 s'attaquant respectivement à 12 (48,0%), 12 (48,0%), 7 (28,0%) et 1 (4,0%) isolats bactériens, tandis que STU ne s'attaquait à aucun des isolats bactériens.
Conclusion: Cette recherche présente la première caractérisation moléculaire de *Bdellovibrio* spp au Nigéria, servant ainsi d'étude de base pour les futures recherches sur *Bdellovibrio* au Nigéria. Elle met également en évidence le potentiel prometteur de Bdellovibrio spp dans le contrôle de *K. pneumoniae* et *Enterobacter* spp associés aux infections chroniques des plaies.

Mots-clés: *Bdellovibrio*; résistance multiple aux médicaments; *Klebsiella*; *Enterobacter*; plaie; bactéries prédatrices

Introduction:

The increasing rise in the emergence of multidrug-resistant (MDR) clinical pathogens has saddled the health sector with a huge burden, especially in developing countries that require better regulatory policies on the use of antibiotics (1). This resistance is mostly exhibited by the notorious ESKAPE pathogen group comprising Gram-negative and Gram-positive organisms. The Gram-negative members include *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterobacter* spp (2,3). These are implicated in many difficult-to-treat bacterial infections like chronic wound infections (4) and often lead to extended hospitalisation and mortality in some complicated cases (5). To this end, the search for innovative alternatives and workable treatment methods that can effectively curb the menace caused by these antibiotic-resistant pathogens, especially in clinical settings remains critical (6,7). As such, many approaches including microbial predation have been suggested as possible alternatives (8).

Microbial predation is the interaction between two microorganisms where one interacting organism (predator) preys on the other, referred to as prey or host. This is an age-long natural phenomenon that serves to bring equilibrium to microbial ecosystems, which helps impact diversity and balance within bacterial communities in different ecological habitats (9,10). It is widely observed in microbes such as viruses (bacteriophages), protozoa, and bacteria. Bacterial predation has been extensively studied by various authors, and some predatory bacteria such as *Bdellovibrio* spp, *Myxococcus xanthus, Vampirococcus,* and the recently discovered *Bradymonabacteria* have been reported (9,11-13).

Bdellovibrio and Like Organisms (BALOs) are a group of obligate, aerobic, rod-shaped Gram-negative predatory bacteria. They predate other Gram-negative organisms as the sole source of nutrients (9). They are highly ubiquitous and have been isolated from various environmental sources since the first isolation of its representative member, *Bdellovibrio bacteriovorus* from the soil in 1963 (14). Their habitats include but are not limited to fresh and salt waters (15,16), soil (17), gastrointestinal tracts of animals (18) and even the human gut (19). *Bdellovibrio* spp are highly motile uniflagellate bacteria with sizes ranging between 0.3–0.5µm by 0.5–1.4µm (20). They employ different predation strategies, the most common of which is endobiotic predation which involves invading the periplasmic space of the prey and depleting its nutrients before host cell lysis.

The life cycle of *Bdellovibrio* spp is usually host-dependent and occurs in three stages; (i) the attack phase, (ii) the invasion stage and (iii) the septation and lysis phase (11,21). In the attack phase, the predator

uses its flagellum to seek and collide with its prey and attach to it using the type IV (type IVa and IVb) pili (22). After this, *Bdellovibrio* invades the periplasm of the prey by forming pores on the peptidoglycan, leading to an alteration and formation of a rounded figure called bdelloplast in the invasion phase. The final phase involves septation and lysis where *Bdellovibrio* uses the nutrients of the prey to undergo replication, followed by synchronous septation into an odd number of daughter cells before it finally lyses the bdelloplast. The *Bdellovibrio* progeny then bursts open from the pores to complete their elongation process before starting a new attack phase on nearby host cells (23,24).

The genus *Bdellovibrio* has been well reported to possess predatory ability on several Gram-negative bacteria in various fields of research. *Bdellovibrio bacteriovorus* has been used in the environment to control the microbial load of *Salmonella* Typhi (the aetiologic agent of typhoid fever) in water (25). In another study (26), the same species was reported to prey on bacterial isolates (*Stenotrophomonas maltophilia* N036 and *Sphingomonas paucimobilis*) from activated sludge flocs in wastewater leading to a 1000-fold reduction of the prey cells in colony forming units (CFU/ml). A study conducted in 2020 reported the ability of *B. bacteriovorus* to prey on the causative organism of potato root rot, *Pectobacterium atrosepticum* (27). The healthcare sector is also a huge beneficiary of studies reporting the predation ability of *B. bacteriovorus* on many Gram-negative bacteria associated with severe infections. Bacterial isolates recovered from oral (*Aggregatibacter actinomycetemcomitans* and *Eikenella corrodens*) and ocular (*Serratia marcescens* and *P. aeruginosa*) infections have been found susceptible to predation by *B. bacteriovorus* (6,28,29). Again, successful predation on cystic fibrosis isolates by the species *B. bacteriovorus* has also been reported (30,31).

Despite the extensive number of studies reporting the isolation and potential applications of *Bdellovibrio* spp from various sources across the globe, no study has reported the isolation and subsequent molecular characterization of *Bdellovibrio* species in southwest Nigeria. The only report of the occurrence of *Bdellovibrio* spp in Nigeria was carried out in Benue State in the middle belt region (15). Presently, no study exists on the molecular characterization of *Bdellovibrio,* and their potential use in the treatment of clinical Gram-negative infections in Nigeria. Therefore, this study was designed to isolate *Bdellovibrio* spp from different environmental samples, characterize the isolates using molecular techniques, and evaluate the prey range and *in-vitro* predatory capability on *K. pneumoniae* and *Enterobacter* spp isolated from chronic wound infections. This study will serve as a baseline study to understand the diversity of *Bdellovibrio* species in Nigeria and possibly harness their predatory potentials to treat microbial infections of chronic wound.

Materials and method:

Sample collection:

A total of fifteen environmental samples, comprising 10 water (8 pond-water and 2 wastewater) samples, 3 soil and 2 faecal samples, including cow dung and chicken faeces were collected from different areas in Osogbo, southwest Nigeria between November 2022 and April 2024 (Fig 1a). The pond and wastewater samples were collected in sterile 250 ml reagent bottles using aseptic techniques. A 100 g of soil sample was collected at a depth of about 25-30 cm in the ground, placed in sterile plastic Ziplock bags and labelled appropriately.

Faecal samples were collected in sterile polythene bags. A detailed map of the sampling sites is shown in Fig 1a, while images of two sites are shown in Fig 1b. All the samples were transported in ice boxes to the Microbiology laboratory of the Osun State University and processed using standard microbiological techniques for the isolation of *Bdellovibrio* spp within 4 hours of collection.

Isolation and characterization of *Bdellovibrio* spp from environmental samples:

Host preparation for isolation of Bdellovibrio

Bdellovibrio spp are mostly isolated in their host-dependent stage hence the need for a Gram-negative bacterium to serve as a host for the isolation. Two *Klebsiella pneumoniae* isolates*,* one obtained from the *Bdellovibrio* research team of the Department of Biotechnology, Osun State University, and the second recovered from a chronic wound sample in a previous study (32) were used as the bacterial prey in the preliminary isolation of *Bdellovibrio* strains.

The bacterial prey was initially cultured on MacConkey agar, then 2-3 pure colonies on solid media were inoculated in Nutrient Broth (NB) for 24 hours at 37°C, harvested by centrifugation at 5000rpm for 10 minutes and washed twice with 25 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2-7.4). The supernatant was discarded, and the resulting pellet, suspended in HEPES buffer, was used for the isolation.

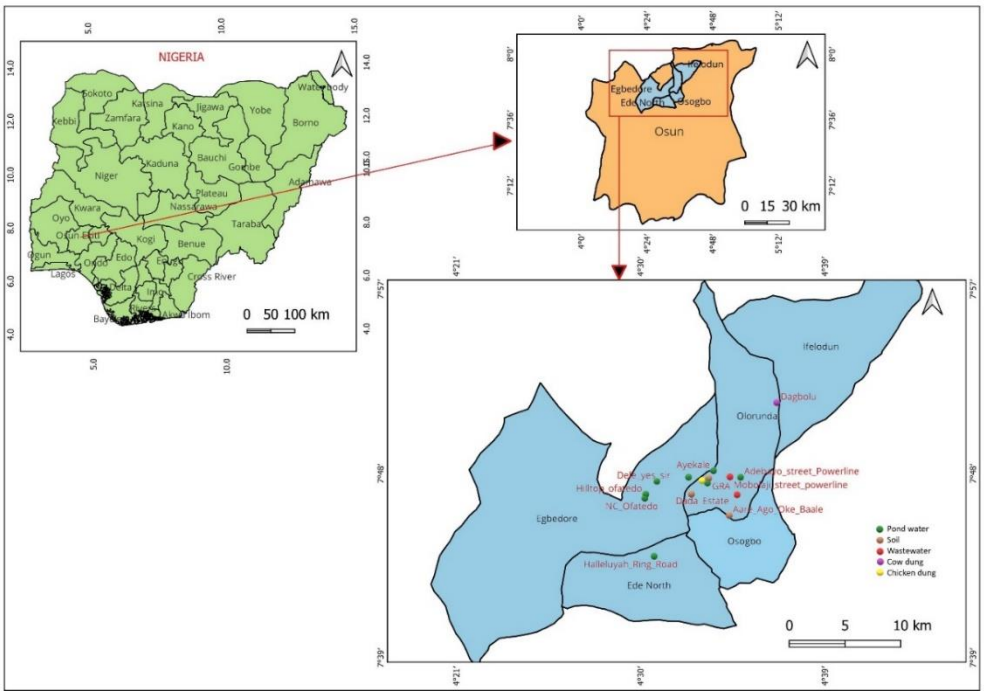

Fig 1a: Map of the study area showing the different sampling sites

Fig 1b: Some sites from which (i) soil, and (ii) pond water samples were collected for the isolation of *Bdellovibrio* spp within Osogbo metropolis, Nigeria

Isolation of Bdellovibrio strains:

The isolation of *Bdellovibrio* was done using previously described methods (17,33-34) with modifications. Briefly, an enrichment step was performed before plating, and dilute nutrient broth (DNB) agar was used to isolate *Bdellovibrio* spp. from the water samples. A 100 ml aliquot of each water sample was centrifuged at 5000 rpm for 10 minutes twice consecutively and filtered using a 0.45 µm sterile syringe filter (ThermoFisher Scientific).

For the soil and faecal waste samples, 100g of each was suspended in sterile HEPES buffer, shaken for an hour at 200 rpm in a shaker incubator, followed by centrifugation and filtration of the suspension. The filtrates were then added to 100 ml of washed prey cells (described in 2.2.1. above) in HEPES buffer and incubated for 72 hours. Afterwards, the enriched cultures were centrifuged, filtered and the filtrates were serially diluted up to 10^9 in sterile HEPES buffer. The different dilutions

of the filtrates (450µl) were then used to re-suspend washed pellets of the prey cells in sterile Eppendorf tubes. These were then plated on dilute nutrient broth (DNB) agar using the double-layer agar plating technique.

The 450µl suspension containing potential predators and the prey was added to 4 ml of molten DNB top agar (0.08% nutrient broth amended with 3ml of 3mmol/L $MgCl_2$. $6H_2O$, 6ml of 2mmol/L $CaCl_2.2H_2O$ and 0.6% agar at pH 7.2) cooled to 45℃, mixed by light shaking and gently overlaid on DNB bottom agar (same composition as DNB top agar except for agar which is 1.9%). The plates were incubated at 30℃ for 7 days and monitored for plaque formation after every 12 hours.

Plaque formation within 48 hours, with a subsequent steady increase in the size of plaques per day was presumed to be potential *Bdellovibrio.* These plaques were further purified using the single plaque isolation technique described by Jurkevitch (33). The purified lysate was stored in sterile glycerol at -80°C.

Phenotypic characterization of Bdellovibrio

The isolated *Bdellovibrio* strains were phenotypically characterized based on plaque morphology and microscopy. Light and scanning electron microscopy (SEM) were employed to visualize the cells. Light microscopy was carried out using the hanging drop method to identify *Bdellovibrio* spp based on motility and size in comparison to the host. *Bdellovibrio* spp were also examined under SEM to visually observe the cells based on size and shape.

***Molecular identification and characterization of Bdellovibrio* spp by PCR and sequencing**

Phenotypically identified *Bdellovibrio* spp were genotypically confirmed by conventional PCR using *Bdellovibrio* genus-specific primers. DNA extraction was done using Daan Gene RNA/DNA purification kit (Spin Column) (Daan Gene Co., China) according to the manufacturer's instructions. The 16S rDNA and *hit* locus genes were amplified using the primer pairs and PCR conditions (Table 1) previously described (17).

Each PCR reaction was carried out in a thermocycler Nexus Gradient 230 (Eppendorf, Germany) and consisted of 12.5µL of 2× Master-Mix (Biolabs, England), 1µL of each 10 µM primer (forward and reverse), 5µL of the DNA template, made up to 25µL with 5.5µL DNAse/RNAse free sterile water. The PCR amplicons were detected by gel electrophoresis using 1% w/v agarose stained with 1.0% Safe View Classic at 80V for 60 min along with 100 bp DNA molecular weight standard (Biolabs, England). The gel was visualized using the UV trans-illuminator E-BOX-CX5 TS imaging system (Vilber, France).

The 16S rDNA amplicons were sequenced using the Sanger sequencing method, with the same primer pair described above. The sequencing chromatogram was edited and cleaned using FinchTV software (https://finchtv.software.informer.com/1.4/) and a similarity search was carried out using BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nml.nih.gov/Blast.cgi). A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) v11 software with the neighbour-joining (NJ) algorithm to recover their clustering pattern and to show the relatedness of the recently isolated *Bdellovibrio* spp with previously reported strains. Bootstrap values were obtained by conducting 1,000 replicates.

Analysis of prey range of *Bdellovibrio* spp

The double-layer agar plating method used to isolate *Bdellovibrio* spp. was applied to determine the prey range of the recovered *Bdellovibrio* spp. Twenty-five antibiotic-resistant Gram-negative bacterial isolates made up of strains of *K. pneumoniae* (n=6) and *Enterobacter* spp (n=19) obtained from diabetic and other chronic wound samples of hospitalized patients in our previous study (32) were selected for the prey range analysis.

The bacterial prey was cultured on MacConkey agar plates for 24-48 hours at 37°C and then inoculated into the nutrient broth. The prey range experiment was carried out in triplicates and plaque formation was monitored for at least 7 days.

Table 1: Primers and PCR conditions used for molecular identification of *Bdellovibrio* spp

Primer Name		Oligonucleotide sequences	PCR conditions (30 cycles)				
			Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
16S rDNA I (492 bp)	Bbs F216	5′ TTTCGCTCTAAGATGAGTCCGCGT 3′	95°C for 5 mins	95°C for 30 secs	57.3°C for 30 secs	72°C for 40 secs	72°C for 10 mins Hold: 4°C ∞
	Bbs R707	5′ TTCGCCTCCGGTATTCCTGTTGAT 3′					
hit locus (959 bp)	Bd *hit* F	5′ TCTAGACAGATGGGATTACTG 3′			58.5°C for 30 secs		
	Bd *hit* R	5′ GAATTCTGGCATCAACAGC 3′					

Results:

Isolation of *Bdellovibrio* spp

In this study, five strains of *Bdellovibrio* were isolated and phenotypically identified based on their ability to form irregular plaques which increased in size steadily within 48-72 hours of incubation.

The isolated strains, designated UAB, PW2, PW5, PW6 and STU, were recovered from pond water (UAB, PW2, PW5 and PW6) and soil sample (STU). Fig 2 shows the characteristic plaque formation by *Bdellovibrio* spp on DNB agar after 48 hours (Fig 2A), and the size change after 72 hours (Fig 2B).

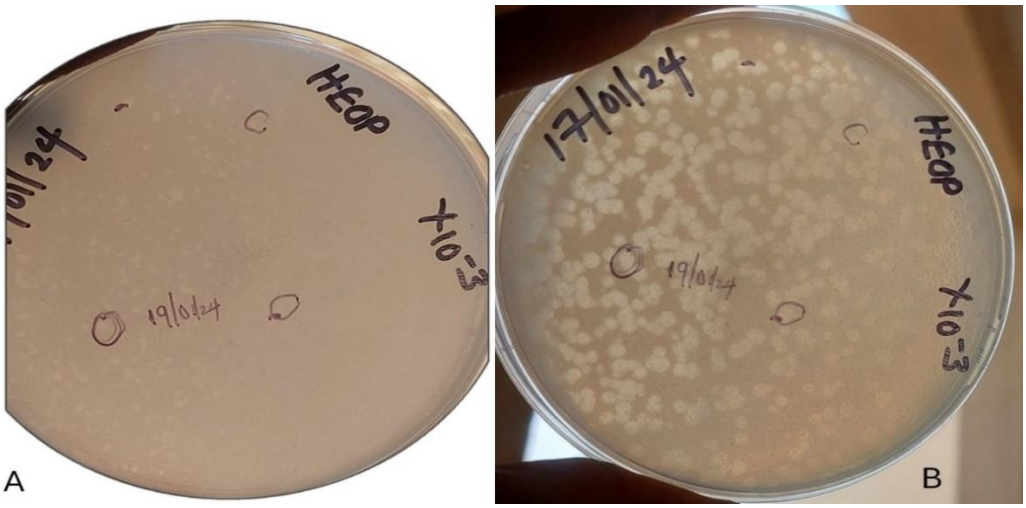

Fig 2: Plaque formation by *Bdellovibrio* PW2 after 48 hours co-cultured with *Klebsiella pneumoniae* on DNB agar plate at 30°C after a) 48 hours post incubation and b) increased size at 72 hours post incubation

Fig 3: The Scanning Electron Micrograph showing *Bdellovibrio* UAB in a co-culture with *K. pneumoniae*. The white arrow indicates the small comma-shaped *Bdellovibrio* spp. and the black arrow shows rod-shaped *K. pneumoniae*, serving as the prey

Characterization of *Bdellovibrio* spp by light and scanning electron microscopy (SEM):

The live image on light microscopy revealed the presence of active and highly motile Gram-negative rods which dashed across the field of view when present. Scanning electron microscopy (SEM) also revealed the finer details of the cellular structure, shape and size of the *Bdellovibrio* spp (Fig 3).

The SEM image revealed the presence of the prey bacteria and the predator in a co-culture. In Fig 3, the black arrow indicates the long, tiny rod *K. pneumoniae* which served as prey, while *Bdellovibrio* strain UAB (marked by the white arrow) is seen as a small comma-shaped predator which appears smaller than the prey.

Molecular characterization of *Bdellovibrio* spp

The 16S rRNA conserved region of the *Bdellovibrio* spp was amplified in all the strains with a band size of 492 bp (Fig 4). However, the *hit* locus was not amplified in any of the strains. The genome sequences searched in NCBI and all the *Bdellovibrio* species were found to be members of this Bdellovibrionae family in the genus *Bdellovibrio*.

All the recovered *Bdellovibrio* species clustered within the same *Bdellovibrio* genus on the phylogenetic tree. The strains STU, UAB and PW5 clustered with 11% identity to each other, with PW5 clustering with an independent branch to *Bdellovibrio* sp Kdesi (accession number-MG957118.1) with 3% identity. STU and UAB clustered together with 19% identity and collectively to *Bdellovibrio* spp CKG001 (accession number-LC797339.1). Also, *Bdellovibrio* spp PW6 and PW2 clustered with a hitherto uncultured *Bdellovibrio* spp clone 5WJ (accession number-MF806547.1) and *Bdellovibrio* spp TY (accession number-GQ410764.1) with 41% and 67% identity respectively (Fig 5).

The 16S rDNA gene sequence data obtained for all the isolated *Bdellovibrio* spp have been submitted to GenBank databases under accession numbers PP826746.1, PP826747.1, PP826748.1, PP826749.1 and PP826750.1 for *Bdellovibrio* strains STU, UAB, PW2, PW5 and PW6 respectively. Details of the data submission can be found at GenBank: https://www.ncbi.nlm.nih.gov/

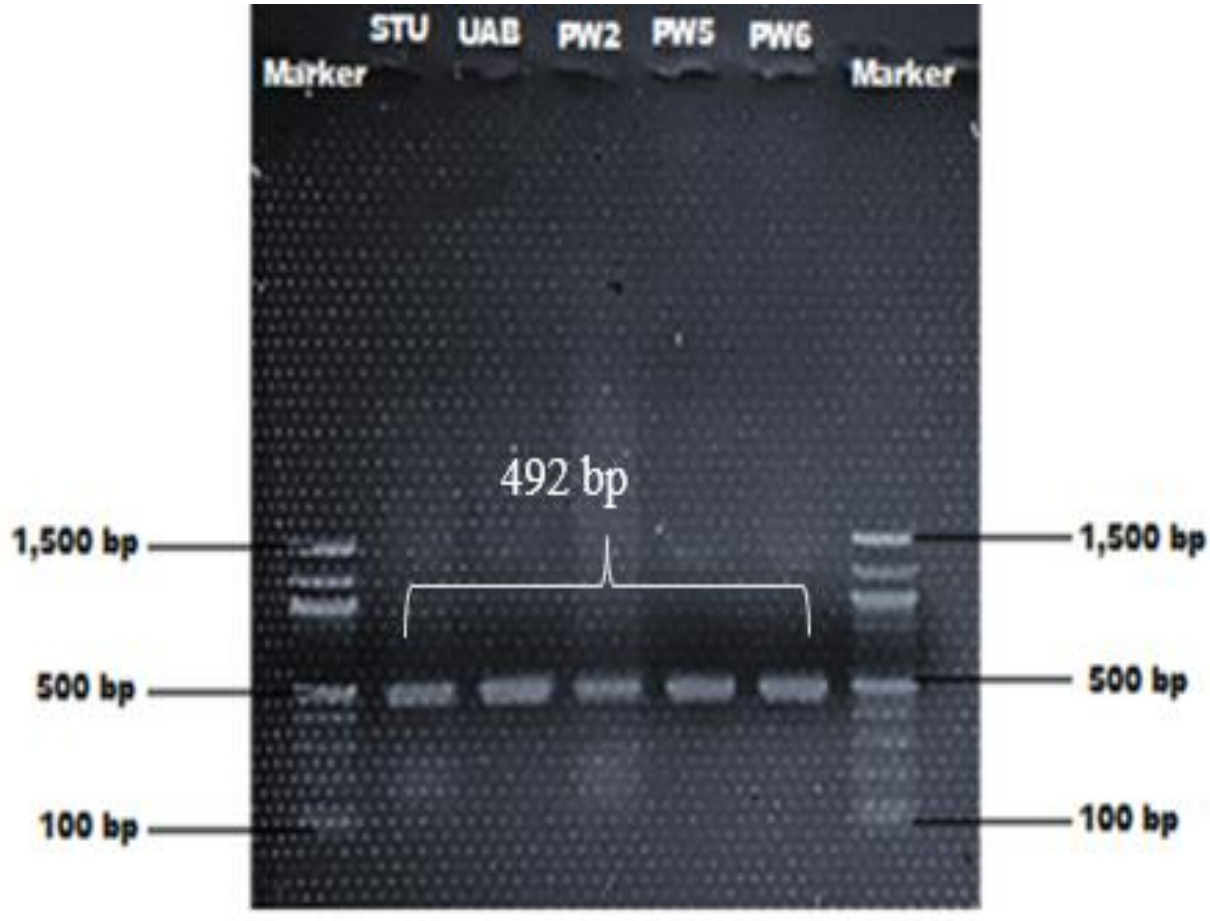

Fig 4: Gel electrophoresis of amplified specific 16S rDNA gene of *Bdellovibrio* spp. amplified at 492 bp. Lanes 1 and 7 - 100 base pair DNA molecular marker; Lanes 2 - 6: DNA band of *Bdellovibrio* sp. (STU, UAB, PW2, PW5, and PW6)

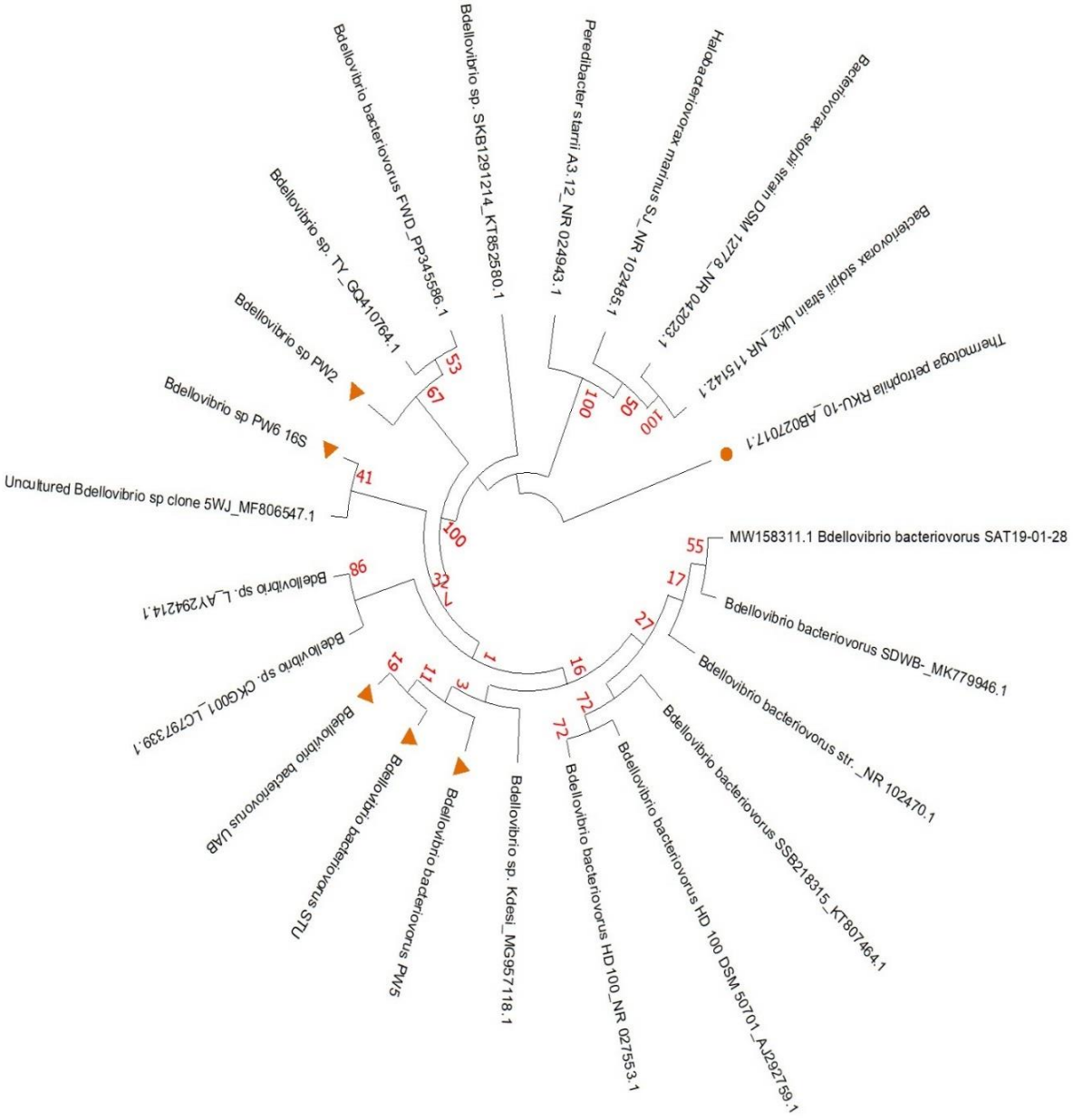

Fig 5: The Phylogenetic tree of the partial sequencing of the 16S rDNA gene in *Bdellovibrio* spp. The tree was constructed using the Neighbor-Joining algorithm in MEGA11. The bootstrap test values (in percentage) out of 1000 replicates in which the associated taxa clustered are shown next to the branches. *Thermotoga petrophila* 16S rDNA sequence was used as the external group to root the tree

Prey range of isolated *Bdellovibrio* spp:

The prey range of the isolated *Bdellovibrio* spp was established and successful predation by some of the *Bdellovibrio* strains was recorded in the study. *Bdellovibrio* strains UAB and PW6 formed plaques with 12 isolates. *Bdellovibrio* UAB preyed on 9 *Enterobacter* spp and 3 *K. pneumoniae* while *Bdellovibrio* PW6 preyed on 8 *Enterobacter* spp and 4 *K. pneumoniae.*

Furthermore, *Bdellovibrio* PW5 and PW2 preyed upon seven isolates (5 *Enterobacter* spp, 2 *K. pneumoniae*) and one isolate (1 *K. pneumoniae*) respectively. Meanwhile, no plaque formation was recorded for STU on any of the tested isolates. The number of plaques formed on the plates varied between 2 to 8 for PW6, and 8 to uncountable for UAB. *Bdellovibrio* PW5 formed plaques too numerous to count on all the isolates it preyed upon. Details are shown in Table 2.

Table 2: Analysis of the prey range of recovered *Bdellovibrio* strains on selected bacterial isolates

S/N	Prey code	Prey identity	No of plaques				
			UAB	**STU**	**PW2**	**PW5**	**PW6**
1	OS1b	*Enterobacter* spp	-	-	-	+ (TNTC)	-
2	OS03	*Enterobacter* spp	-	-	-	-	-
3	OS04	*Enterobacter* spp	+ (TNTC)	-	-	+ (TNTC)	-
4	OS06	*Enterobacter* spp	+(TNTC)	-	-	-	-
5	OS14	*Enterobacter* spp	+ (15)	-	-	+ (TNTC)	+ (4)
6	OS16	*Enterobacter* spp	-	-	-	-	-
7	OS17	*Enterobacter* spp	-	-	-	-	+ (8)
8	OS20a	*Enterobacter* spp	+ (13)	-	-	-	
9	OS20b	*Enterobacter* spp	-	-	-	+ (TNTC)	+ (6)
10	OS21	*Enterobacter* spp	-	-	-	-	-
11	OS26	*Enterobacter* spp	+ (12)	-	-	-	+ (2)
12	OS28	*Enterobacter* spp	+ (10)	-		-	+ (5)
13	OS29	*Enterobacter* spp	-	-	-	-	-
14	OS34	*Enterobacter* spp	+ (13)	-		+ (TNTC)	-
15	OS37	*Enterobacter* spp	-	-	-	-	-
16	OS38	*Enterobacter* spp	+ (12)	-	-	-	-
17	OS41	*Enterobacter* spp	+ (8)	-	-	-	+ (4)
18	OS42	*Enterobacter* spp	-	-	-	-	+ (5)
19	OS67	*Enterobacter* spp	-	-	-	-	+ (2)
20	OS54b	*K. pneumoniae*	+ (TNTC)	-	-	-	-
21	OS94	*K. pneumoniae*	+ (25)	-	-	-	+ (6)
22	OS115	*K. pneumoniae*	+ (10)	-	+	+ (10)	+ (7)
23	OS122b	*K. pneumoniae*	-	-	-	-	-
24	OS123b	*K. pneumoniae*	-	-	-	-	+ (4)
25	OS137	*K. pneumoniae*	-	-	-	+ (8)	+ (6)
Total			**12 (48.0%)**	**0**	**1 (4.0%)**	**7 (28.0%)**	**12 (48.0%)**

TNTC: Too Numerous to Count

Discussion:

Bdellovibrio spp are predatory bacteria that prey on Gram-negative bacterial species. They act as ecological balancers that control the population of microbial communities (35-36). They also have the potential to be used as biocontrol anti-infective agents against many pathogenic bacterial species associated with infections such as periodontitis and ocular infections (6,37-39). *Bdellovibrio* species are ubiquitous and well-isolated in many environments in different countries. However, there is a dearth of information on *Bdellovibrio* isolation and characterization in Nigeria.

Our study successfully isolated five different *Bdellovibrio* strains from pond water and soil samples. The isolation agrees with the first report of isolation of *Bdellovibrio bacteriovorus* by Stolp and Starr (14) and subsequent other studies in different parts of the world. Oyedara et al., (17) reported the isolation of two different strains of *Bdellovibrio* from agricultural soil sites in Mexico. Another study in Iran also reported the isolation of *Bdellovibrio* spp from the soils of various farm sites (40). Pond waters have also been described as suitable habitats for *Bdellovibrio* spp (41-42). Initial enrichment of the samples with the preferred prey before plating allows for the multiplication of the number of *Bdellovibrio* cells present in the samples (if any).

Dilute Nutrient Broth (DNB) medium used in isolation is an ideal choice because the low nutrient content in the composition prevents the rapid growth of the prey before an attack by the predator. Furthermore, adding hydrated magnesium and calcium chloride to the DNB medium aids the attachment of *Bdellovibrio* spp to the prey cells thereby enhancing predation. To the best of our knowledge, this study will be the first to report the isolation of *Bdellovibrio* spp from environmental samples in Southwest Nigeria.

The genetic characterization of an organism provides insight into its identity and relatedness with other existing cohorts within or outside the same genus. It was observed that all the isolates provided the desired amplicon size, confirming their identity as *Bdellovibrio* spp. The amplification of the 16S rRNA fragment conserved for *Bdellovibrio* species in the recovered species was similar to the findings of Oyedara et al., (17). However, the *hit* locus was not successfully amplified in the isolated *Bdellovibrio* spp in our study. This observation deviates from the reports of other studies (17,34). The *hit* locus is a gene that allows the continuation of the lifecycle of *Bdellovibrio* from the attack to the growth phase. It is proposed as a conserved region in *B. bacteriovorus* (18) and can be used to identify *B. bacteriovorus*. However, possibilities of mutations/deletions in the genome and *hit* locus of the isolated species exist (43), and the probability of intrinsic variations in the genomic constitutions of strains across geographic regions is also feasible as these strains have hitherto never been previously isolated and characterized in the present study area. Therefore, whole genome sequencing (WGS) may provide more salient genetic data on the

conserved nature of the *hit* locus of the newly isolated strains if present.

Furthermore, our study observed different clustering patterns among the *Bdellovibrio* spp. The *Bdellovibrio* strain (STU) isolated from a soil sample clustered closely with other species from a different source and sample site. It clustered with isolates UAB and PW5 recovered from pond water in this study and also *Bdellobvibio* spp Kdesi previously isolated from sewage in Mexico by Guo et al., in 2018 (unpublished strain). *Bdellovibrio* strain PW2 clustered with *Bdellovibrio* spp TY (acession number-GQ410764.1) with 67% identity. Meanwhile, *Bdellovibrio* strain PW6 clustered with an uncultured *Bdellovibrio* spp clone 5WJ (MF806547.1) isolated from groundwater.

Generally, it was observed that marine BALOs such as *Peredibacter starrii*, *Bacteriovorax stolpii* and *Halobacteriovorax marinus* SJ clustered farther and separately on the phylogenetic tree. This aligns with earlier reports of Jurkevitch et al., (33) and Baer et al., (44) which explained the distant relationship that exists between the marine BALOs (which belong to the family Bacteriovoraceae) and terrestrial BALOs (family Bdellovibrionaceae). The two groups were formally grouped as one but later separated based on differences in characteristics like prey preference and response to salinity. However, further genomic analysis may provide more insights.

Chronic wounds are frequently infected by MDR Gram-negative ESKAPE pathogens that defy treatment by conventional antibiotics using different resistance mechanisms. This requires new and safe alternatives to curb the menace of these pathogens and predation by *Bdellovibrio* spp is proposed as a viable and effective alternative. The activity of the *Bdellovibrio* isolates evaluated against 25 clinical isolates (*K. pneumoniae* and *Enterobacter* spp) recovered from chronic wounds in our study shows that 2 of the *Bdellovibrio* isolates (UAB and PW6) exhibited a higher predation rate of 48.0% on the selected isolates compared to strains PW2 and PW5, which only exhibited 4.0% and 28.0% rates. This agrees with previous reports (5,34,45-46). Sun et al., (5) observed a 65.2% predation efficacy of *B. bacteriovorus* on clinical isolates of *K. pneumoniae*. Suresh and his team (34) in India recorded predation on another *P. aeruginosa* strain (ADW44) recovered from a diabetic wound ulcer. Furthermore, Tajabadi et al., (46) reported *in-vivo* predation of *P. aeruginosa* strain PAO by *B. bacteriovorus* in a burn wound-induced study.

Most studies to determine the predation efficacy of *Bdellovibrio* spp have used Gram-negative bacteria such as *K. pneumoniae*, *P. aeruginosa*, *Escherichia coli* and even *Salmonella* spp as bacterial prey recovered from wounds without including *Enterobacter* spp. However, our study included *Enterobacter* spp and recorded successful predation on some species. Therefore, the prey range result of the isolated *Bdellovibrio* spp in our study against MDR *K. pneumoniae* and *Enterobacter* spp recovered from chronic wounds further buttresses their therapeutic potential in treating wound infections caused by these pathogens.

Additionally, it was observed that in this study, the prey range pattern for the *Bdellovibrio* spp was not consistent because they did not prey on the same set of organisms except for two isolates [OS14 (*Enterobacter* spp) and OS115 (*K. pneumoniae*)] which were preyed upon by all the *Bdellovibrio* strains. Although resistance to predation has not been reported, this result suggests that selective or preferential predation may exist. The first stage in the life cycle of a prey-dependent *Bdellovibrio* is the attachment stage. At this stage, it is expected that *Bdellovibrio* finds a prey and attaches to it usually reversibly in the first few minutes until it decides the suitability of the prey. When *Bdellovibrio* finds the prey susceptible, it irreversibly attaches and penetrates the periplasm to complete predation and if not suitable, it detaches from such prey (47).

It is therefore possible that the non-predation of *Bdellovibrio* on some of the tested prey is due to the non-susceptibility of the prey to predation by the reported *Bdellovibrio* spp. Additionally, resistance to predation may also be genetic such as a change in a single gene in the prey. For instance, Aharon et al., (48) reported a mutation in *manC* which led to an altered O-antigen in *Acidovorax citrulli* and a subsequent increase in the resistance to predation.

Conclusion:

Bdellovibrio spp are obligate predators with biocontrol potentials in different fields of life. The successful isolation of *Bdellovibrio* spp in our study from environmental samples (soil and pond waters) is a pioneer report in southwest Nigeria. This study reports the isolation and 16S rRNA characterization of indigenous *Bdellovibrio* spp from Nigerian environmental samples. It also demonstrated the prey range analyses and potential use of these indigenous *Bdellovibrio* spp as biocontrol agents against strains of *K. pneumoniae* and *Enterobacter* spp which are members of the ESKAPE pathogen group commonly recovered from infected chronic wounds. Therefore, our study can serve as a baseline for future *Bdellovibrio* characterization studies in Nigeria.

Additionally, predation by *Bdellovibrio* spp is not prey-specific, and any susceptible Gram-negative organism can serve as the prey. Therefore, studying prey selection, pre-

dator-prey interaction and resistance to *Bdellovibrio* is essential before applying *Bdellovibrio* spp as a biocontrol agent against a specific pathogen of interest. Summarily, this study shows the diversity of *Bdellovibrio* spp population in the environment. It also confirms the potential application of *Bdellovibrio* to reduce or eliminate antibiotic-resistant *K. pneumoniae* and *Enterobacter* spp in chronic wounds which can help alleviate secondary effects of chronic wound infection like prolonged hospital stay and increased cost of medication.

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Contributions of authors:

AOH was involved in study conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing original draft, review editing, and funding acquisition; OOO was involved in the study methodology, validation, resources, data curation, and manuscript review editing; WAA was involved in the study methodology, validation, resources, data curation, review editing, supervision, and project administration; YONA was involved in the study methodology, validation, formal analysis, investigation, resources, data curation, writing original draft, and manuscript review editing; AFA was involved in study conceptualization, data curation, and funding acquisition; and AFM was involved in study methodology, validation, formal analysis, investigation, resources, data curation, writing original draft, manuscript review editing, supervision and project administration.

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Conflict of interest:

Authors declare no conflict of interest.

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