Journal of Water & Health

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Journal of Water and Health Vol 21 No 2, 147 doi: 10.2166/wh.2023.136

Predatory efficacy of *Bdellovibrio stolpii* isolated from the wastewater sources against the multidrug-resistant clinical isolates

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ABSTRACT

The present study investigated the predatory efficacy of *Bdellovibrio* isolated from untreated hospital wastewater sources against human pathogens. *Bdellovibrio* is a Gram-negative, motile, uniflagellate predatory bacteria present in the environment, which directly predates on other bacteria, including human pathogens. In the present study, 30 hospital effluent samples were collected and screened for *Bdellovibrio*. A total of 11 *Bdellovibrio* isolates were obtained by the double-layer agar technique. All the isolates were identified by amplification of the 16S rDNA coding region using polymerase chain reaction (PCR) and confirmed as *Bdellovibrio stolpii*. The lytic activity of confirmed isolates was investigated against four Gram-negative bacteria *Pseudomonas aeruginosa* ADW44, *Pseudomonas aeruginosa 27853(ATCC Strain)*, *V. cholerae* and *Salmonella typhimurium* of clinical origin obtained from the institutional repository. Among the 11 isolates, three *Bdellovibrio* isolates NBd1, YBd1 and RBd1 demonstrated the ability to prey upon the tested clinical isolates. To the best of our knowledge, this is the first report on the isolation of *B. stolpii* from hospital wastewater settings in India with broad and high bacteriolytic activity.

Key words: antibiotic resistance, *Bdellovibrio*, biofilm, hospital effluent, predation, *Pseudomonas aeruginosa*

HIGHLIGHTS

- Antibiotic resistance in bacteria is a major concern in both national and international scenarios.
- The study gives insights into the predatory spectrum of *Bdellovibrio*, against multidrug-resistant bacteria.
- The present study may help in the development of *Bdellovibrio* as a whole-organism approach against biofilm-forming bacteria.
- Developing more efficient, economic, and environmentally friendly biocontrol agents against multidrug-resistant bacteria is the need of the hour.

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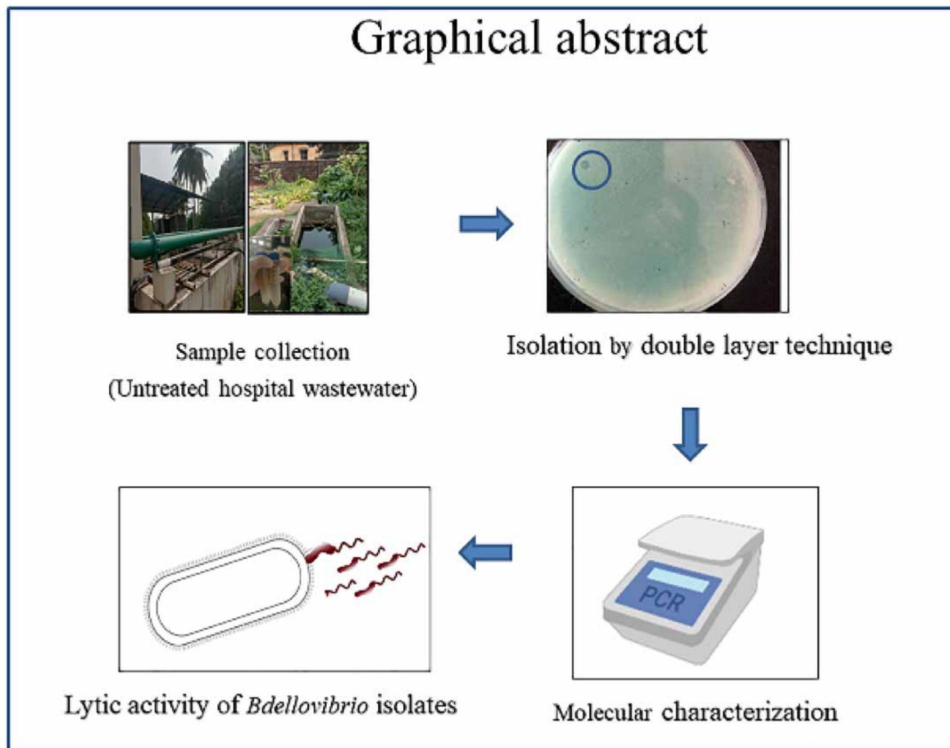
GRAPHICAL ABSTRACT



INTRODUCTION

Growing concerns among a greater number of individuals about the emergence of drug resistance within bacterial communities are being raised due to the extensive use and abuse of antimicrobial agents. In addition to drug resistance, bacterial biofilms being resistant to antimicrobial agents is another threat. The treatment of infections caused by the biofilm formers is a major challenge since these bacteria are protected well inside the biofilm which results in more resistance to antibiotic treatment. Extensive research has revealed its complex mechanism of infection and treatment strategies are limited because of a lack of sensitive detection methods and a narrow range of effective antibiotics. Facing the limitations of antibiotics, there is an increasing need for the development of other alternative methods for the efficient control of clinical pathogens and managing bacterial infections caused by these pathogens. *Bdellovibrio* is a predatory bacterium present in the environment that directly predates on other bacteria which overviews the concept of a biocontrol agent.

Bdellovibrio is a group of obligatory predatory bacteria which prey upon other bacteria especially Gram-negative bacteria for nutrients and reproduction. The organism was first discovered by Stolp & Starr (1963) from the soil during the isolation of *Pseudomonas* bacteriophage. *Bdellovibrio* is a small Gram-negative bacteria measuring 0.2–0.5 μm × 0.5–2.5 μm in size, highly motile with single-sheathed polar flagella (Kadouri & O'Toole 2005). *Bdellovibrio* predates on other larger Gram-negative bacteria by invading their periplasmic space, degrading the prey cellular macromolecules and undergoing a complex replication cycle releasing the progeny (Rendulic *et al.* 2004; Caulton & Lovering 2020). They exhibit a dimorphic cell cycle with several events such as prey location, attachment, penetration, establishment, elongation, development (septa formation) and progeny release (Lambert *et al.* 2006). The lytic action of the *Bdellovibrio* species rapidly reduces prey populations.

Bdellovibrio species are ubiquitous in nature and are found in different environmental sources such as soil, freshwater, rhizosphere and the gastrointestinal tract of animals (Sar *et al.* 2015; Oyedara *et al.* 2016). The strains also have the capacity to grow in the absence of prey as well as in the presence of nutrient-rich media using laboratory protocols (Ferguson *et al.* 2008). The study provided an important insight into the significance of predatory bacteria in the shaping of community structure, its impact on complex communities and its function in both natural and engineered ecosystems (Feng *et al.* 2016). The predation

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efficiency of *Bdellovibrio* was evaluated against six different periodontal pathogens (Van Essche *et al.* 2011). The strain *Bdellovibrio* (HD100) markedly reduced the biofilm formation and was used to combat periodontal pathogens (Van Essche *et al.* 2009).

Bdellovibrio with several prospective applications as biocontrol agents in aquaculture, animal husbandry and medicine is gaining momentum considering the increasing trend of antibiotic resistance among pathogenic bacteria and the use of living predatory bacteria is one of the potential approaches to overcome this global health and economic crisis (Atterbury & Tyson 2021). Even though interest in predatory bacteria has recently surged, only *Bdellovibrio bacteriovorus species* is studied best among BALOs (*Bdellovibrio*-like organisms), and serves as a model organism for bacterial predation (Bratanis *et al.* 2020). Many of the molecular mechanisms during prey invasion, mode of nutrient acquisition, and details on their importance of predation remain partial and rather elusive. The emergence of newer multidrug-resistant (MDR) pathogens pays a severe burden on the health infrastructure of developing countries. There is a dearth of local findings on *Bdellovibrio* in India and their predatory activities against clinical isolates to use them as a whole organism approach.

Bdellovibrio strains exhibit obligatory lifestyle and selectively prey on a broad range of Gram-negative bacteria, including MDR pathogens. The kinetic lysis of the *Bdellovibrio* as a predator to antibiotic-resistant *Salmonella paratyphi* as prey organisms was demonstrated and suggested their use of biocontrol agents in water (El-Shanshoury *et al.* 2016). Most of the published research used pure cultures of *Bdellovibrio bacteriovorus* to elucidate the prey–predator interaction. The predatory behavior of these *Bdellovibrio* in complex natural habitats is still to be studied. Therefore, this study would significantly contribute to the existing database of information on *Bdellovibrio* and thereby may help in bringing a step closer to the eventual widespread applications of *Bdellovibrio stolpii* for the treatment of infections in man. The isolation of *Bdellovibrio* from different environmental sources like soil and water was reported in previous studies (Rogosky *et al.* 2006; Markelova & Kerzhentsev 1998; Chu & Zhu 2010). Predatory activities of isolated *Bdellovibrio* were evaluated against periodontal pathogens and aquaculture isolates (Van Essche *et al.* 2011). *Bdellovibrio* has more advantages over bacteriophages with multiple preys and the capability to invade biofilms. Dual predation by bacteriophage and *Bdellovibrio* in the eradication of *Escherichia coli* prey was more successful where individual predation cannot (Hobley *et al.* 2020). Detection of *Bdellovibrio* in hospital wastewater and evaluation of predatory efficacy against the clinical isolates has not been elaborately elucidated before. The present study would therefore give insights into the predatory spectrum of isolated *Bdellovibrio* which may help in the development of *Bdellovibrio* as a whole organism approach in treating the infections caused by biofilm-forming clinical pathogens. Hence, the present study was undertaken to isolate *Bdellovibrio* from untreated wastewater samples and to evaluate the inhibitory activity against selected Gram-negative clinical pathogens obtained from the institutional repository.

MATERIALS AND METHODS

Sample collection

Sterile 100 ml containers with stoppers were used for sample collection. Untreated wastewater samples were collected from two tertiary care hospitals located in and around Mangaluru (from the outlet pipe before the treatment). A total of 30 effluents were collected during the study period from December 2020 to June 2021 and processed for *Bdellovibrio* isolation within 4 h after sample collection.

Prey cell preparation for *Bdellovibrio* isolation

Most of *Bdellovibrio* is found as host-dependent growth in nature. Hence, host-dependent isolation technique was used to isolate the *Bdellovibrio* strains. *Pseudomonas aeruginosa* ADW44 from the institutional repository was used as prey culture for the primary isolation of *Bdellovibrio*. *P. aeruginosa* ADW44 is a wound isolate obtained from a diabetic wound (institutional ethical clearance previously obtained). Figure 1 presents the sample collection sites of the tertiary care hospital wastewater treatment plant.

Isolation of *Bdellovibrio*

The untreated hospital wastewater samples collected were centrifuged at 6,000 rpm for 5 min at 4 °C to remove suspended materials. The resulting supernatant was filtered through a 0.45 μm syringe filter to remove all the larger bacteria. The filtrate was again centrifuged at 12,000 rpm for 20 min at 4 °C to pellet out the *Bdellovibrio*. The supernatant was discarded and the pellets were resuspended in 1 ml of HEPES buffer. 1 ml of this suspension was used for the double-layer agar technique wherein it was mixed with 4 ml of dilute nutrient broth agar (0.7% agar) and 0.2 ml of prey culture and plated on dilute

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Figure 1 | Sample collection sites located in and around Mangaluru.

nutrient agar (1.5% agar). The plates were incubated at 30 °C and observed for 3–7 days for the development of the growing plaques (Oyedara *et al.* 2016). Potential *Bdellovibrio* plaques were defined as those that appear between 48 and 72 h and are observed for increased zone size.

Single plaque isolation

The plaques which appeared between 48 and 72 h were transferred to 5 ml of dilute nutrient broth containing prey cells. This culture was incubated at 30 °C for 48 h to monitor the clearance of the suspension. The clear culture obtained was centrifuged at 8,000 rpm at 4 °C for 5 min to remove prey cells and the supernatant was further subjected to centrifugation at 12,000 rpm for 30 min at 4 °C. This step was repeated twice and the pure culture of *Bdellovibrio* was preserved at −80 °C in 40% glycerol broth (Oyedara *et al.* 2016).

Molecular identification of *Bdellovibrio* isolates

For molecular identification, amplification of 16S rDNA gene and hit locus gene was done using polymerase chain reaction (PCR). DNA was extracted from each *Bdellovibrio* isolate by CTAB (Cetyl trimethyl ammonium bromide) method. Briefly, *Bdellovibrio* isolates were inoculated into 5 ml of dilute nutrient broth with prey cells and incubated at 30 °C for 48 h. The prey cells were removed by centrifugation at 8,000 rpm for 5 min and further centrifuged at 12,000 rpm for 30 min to pellet out *Bdellovibrio* cells. To this pellet, 400 μl of TE buffer and 100 μl of NaCl (5 M) were added and mixed well by vortexing. To this, 50 μl of CTAB was added and incubated at 60 °C for 20 min. After the incubation, 500 μl of chloroform was added, mixed well and incubated at 4 °C for 30 min. The incubated suspension was centrifuged at 12,000 rpm for 10 min and the resulting supernatant was collected into a new centrifuge tube. To the supernatant collected, 500 μl of phenol:chloroform: isoamyl alcohol (25:24:1) was added, mixed well and centrifuged at 12,000 rpm for 5 min. The supernatant thus obtained was transferred to a new centrifuge tube and 500 μl of chloroform was added, vortexed and centrifuged at 12,000 rpm for 5 min. To the supernatant obtained, in a fresh centrifuge tube, 1/10 volume of Na-acetate (50 μl) and 2/10 volume of ice-cold ethanol (1,000 μl) were added to precipitate DNA and incubated in ice for 15 min and centrifuged at 12,000 rpm for 10 min. To the pellet, 500 μl of 70% ethanol was added and centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and tubes were inverted on a paper towel to remove traces of ethanol. The resulting pellet was suspended into 50 μl of elution buffer and stored as final DNA preparation at −20 °C and was used as a template in PCR reaction.

A volume of 30 μl of the reaction mixture was used for PCR to check the presence of 16S rDNA for genus-level identification and further species-level identification was performed using specific primers described by Varon & Shilo (1981). All the PCR reactions were carried out in a PCR system (Bio-Rad, CA, USA, Model: S1000). The details of the primers are listed in Table 1.

Specific amplification was performed using a thermocycler with the following PCR conditions: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C (Bd primer) 45 °C (BdBac, BdSp and BdSr primers)

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Table 1 | PCR primer details used in this study for molecular identification of *Bdellovibrio*

Primers	Sequence	Annealing Temp (°C)	Product size (in bp)
Bd	F: AGAGTTTGATTCTGGCTCAGA R: AGGTGATCCAGCCGCAGGTTC	62	~1,495
Hit	F: GTGGCTTCAAACGGAGTGGA R: ACGACTGTGAACGGCAACG	56	~700
BdBac	F: GCGTGCCTAATACATGCAAG R: AGATAGCTTTTAAGCGATTTGCTCTA	45	~1,200
BdSp	F: GCGTGCCTAATACATGCAAG R: CGGTTTTTTGAGATTGGCTC	45	~1,200
BdSr	F: GCGTGCCTAATACATGCAAG R: CCGAACTGAGGCGCGC	45	~1,200

and 54 °C (Hit primer) for 30 s, extension at 72 °C for 45 s, and final extension at 72 °C for 10 min. The PCR products after amplification were subjected to gel electrophoresis. Briefly, 2% agarose gel, stained with ethidium bromide (0.5 μg/ml) was visualized under ultraviolet light using a gel documentation system (Bio-Rad, CA, USA, Model: Gel Doc^{TM}*XR*+). All positive PCR products were purified and sent for sequencing at Eurofins Genomics India Pvt Ltd, Bangalore. The obtained sequences were compared to gene sequences in GenBank by means of the Basic Local Alignment Search Tool (BLAST) present at the National Centre for Biotechnology Information (NCBI) website (http:// www.ncbi.nlm.nih.gov).

Antibiotic resistance profile of Gram-negative pathogens

Antibiotic susceptibility for the test organisms *Salmonella typhimurium, Vibrio cholerae, P. aeruginosa* ADW44 *and P. aeruginosa 27853* (ATCC strain) was performed using the method described by Bauer *et al.* (1966) on Muller–Hinton agar as recommended by Clinical and Laboratory Standards Institute (CLSI 2017) guidelines. The antibiotics used for the study are tetracycline (30 μg), ampicillin (10 μg), chloramphenicol (30 μg), cotrimoxazole (25 μg), ciprofloxacin (5 μg), imipenem (10 μg), nalidixic acid (30 μg), cefotaxime (30 μg) (HiMedia, India). A fresh culture of isolates was grown in 5 ml of Mueller–Hinton (MH) broth up to turbidity of 0.5 McFarland standards and then it was spread using a sterile cotton swab on well-dried MH agar to prepare a lawn. After gentle air drying, the antibiotic discs were placed on the surface of the medium and incubated for 18–24 h at 37 °C for the appearance of a clear zone. The diameter of the zone of inhibitions around discs was measured and results were interpreted as sensitive or resistant.

Biofilm formation ability assay of Gram-negative pathogens

Biofilm-forming ability assay of the host *S. typhimurium, V. cholerae, P. aeruginosa* ADW44 *and P. aeruginosa 27853*(ATCC strain) was done using crystal violet assay (Kadouri & O'Toole 2005). Briefly, 190 μl of nutrient broth and 10 μl of bacterial culture were added to 96-well microtiter plates. Nutrient broth without bacterial culture was used as a control. Plates were incubated for 24 h at 37 °C. After incubation, wells with culture, as well as control were decanted and washed with 0.85% saline three times. Plates were allowed to dry at room temperature and 200 μl of 1% crystal violet was added to each well followed by 10–15 min of incubation. After incubation, wells are decanted again and washed with 0.85% saline three times. Plates are allowed to dry at room temperature for 15 min. Once dried, 200 μl of 33% glacial acetic acid was added and then transferred to a fresh plate. Absorbance was recorded at 630 nm using an ELISA plate reader and organisms were categorized as biofilm former or non-biofilm former.

Lytic activity the *Bdellovibrio* isolates

The lytic ability of the *Bdellovibrio* isolates was determined against four Gram-negative human pathogens, *S. typhimurium, V. cholerae, P. aeruginosa* ADW44 and *P. aeruginosa 27853*(ATCC strain). Optical density (OD) of the prey cells at a time interval of 24 h for 5 days was measured at 600 nm. To initiate the experiment, 1 ml each of *Bdellovibrio* lysate was inoculated into the prey cell culture with OD, $OD_{600nm} = 1$ in 50 ml dilute nutrient broth. The prey cell culture alone in 50 ml of dilute nutrient broth was kept as a control. The co-cultures of *Bdellovibrio* and prey along with the control were incubated at 30 °C with shaking at 120 rpm. The OD at 600 nm was determined using a spectrophotometer (UV-Vis Biospectrometer,

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Eppendorf) at 24-h time intervals for 5 days. This experiment was carried out for all 11 *Bdellovibrio* isolates on different prey cells in duplicates with similar conditions.

RESULTS

Isolation and identification of *Bdellovibrio*

Out of 30 hospital wastewater samples collected, a total of eleven *Bdellovibrio* isolates were obtained using *P. aeruginosa* ADW44 as a prey organism and clear plaques were observed on the lawn of bacterial culture by double-layer agar methods when overlaid with soft agar incubated at 30 °C for 3–7 days with an increase in the plaque size. Plaques became visible after 72 h of incubation at 30 °C (Figure 2).

Molecular identification of *Bdellovibrio* isolates

Molecular identification of the isolated *Bdellovibrio* was performed by PCR using 16S rDNA primers. All 11 isolates were amplified for 16S rDNA primer with an amplicon size of 1,495 bp and confirmed they belong to the genus *Bdellovibrio* (Figure 3). Representative agarose gel electrophoresis of samples for the *hit* gene is presented in Figure 4. Further species-level identification was performed using species-specific primers. All the 11 isolates were confirmed as *B. stolpii* with a

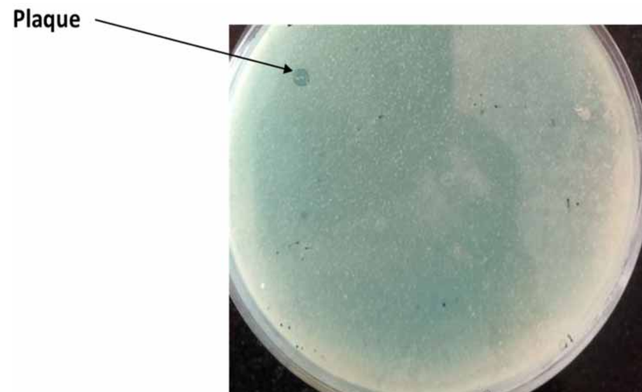

Figure 2 | Typical lytic plaques developed by *Bdellovibrio* on the lawn of *Pseudomonas aeruginosa.*

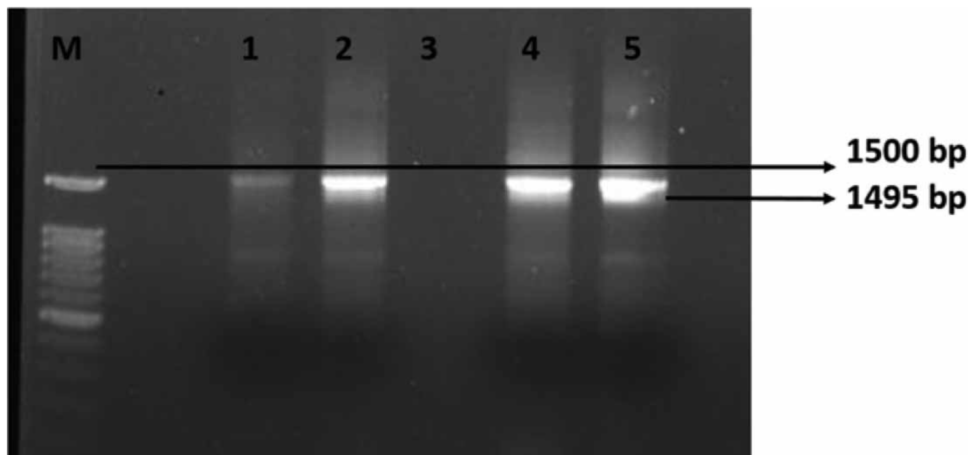

Figure 3 | Representative agarose gel electrophoresis of samples for 16 s rDNA coding region. Lane M: 50-bp marker; Lanes 1, 2, 4, 5: positive samples; Lane 3: Negative sample.

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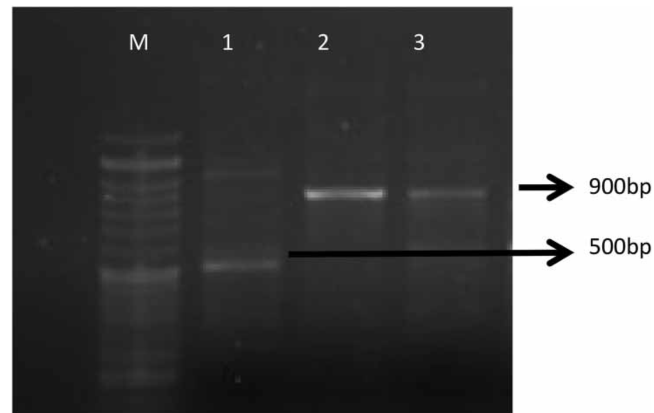

Figure 4 | Representative agarose gel electrophoresis of samples for *hit* gene. Lane M: 50-bp marker; Lanes 1, 2, 3: representative samples.

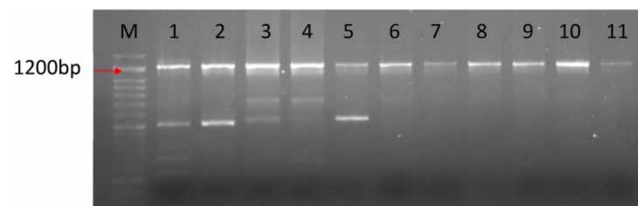

Figure 5 | Representative agarose gel electrophoresis of samples for *Bdellovibrio stolpii* gene. Lane M: 50-bp marker; Lanes 1–11: samples.

product size of 1,200 bp (Figure 5). Among the 11 isolates obtained, all were positive for *B. stolpii*, none showed positive for *B. bacteriovorus* and *B. starii* by PCR. The positive PCR products were confirmed by sequencing and all 11 isolates showed 96–99% similarity with the sequences available in Genbank. PCR confirmed isolates were named as NBd1, YBd1, Ybd2, Ybd3, RBd1, RBd2, RBd3, KHBd1, KHBd2, KHBd3 and KHBd4 based on the site of isolation.

Antibiotic resistance and biofilm-forming ability assay of host bacteria

The details of the antibiotic susceptibility results of the test organisms are presented in Table 2. The zone of inhibition size is provided in the Supplementary Material (S2). Only *Pseudomonas* strains showed MDR pattern as it showed resistance to more than two classes of antibiotics. Biofilm-forming ability of the test results showed that the *P. aeruginosa* ADW44 is a strong biofilm-forming bacteria.

Table 2 | Antibiotic susceptibility pattern of the test organisms

Antibiotic	*Vibrio cholerae*	*Pseudomonas aeruginosa ADW44*	*Pseudomonas aeruginosa 27853*	*Salmonella Typhimurium*
Tetracycline (30 μg)	S	R	R	S
Nalidixic acid (30 μg)	I	R	R	S
Chloramphenicol (30 μg)	S	R	R	S
Ciprofloxacin (5 μg)	S	S	S	S
Cotrimoxazole (25 μg)	S	R	R	R
Cefotaxime (30 μg)	I	S	S	S
Ampicillin (10 μg)	I	R	R	S
Imipenem	S	S	S	R

Note: R, resistant; I, intermediate; S, sensitive.

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Determination of lytic activity of *Bdellovibrio* strains

The activity of 11 isolates of *Bdellovibrio* was checked against *S. typhimurium*, *V. cholerae*, *P. aeruginosa* ADW44, *P. aeruginosa* 27853(ATCC). The graphical representation of predatory activities of all 11 *Bdellovibrio* strains against four clinical isolates are presented in the Figures 6–9. Plaque based assays and monitoring of lysis parameters, and OD determination in broth co-cultures were used for evaluation of the bacteriolytic activity of *Bdellovibrio* strain. Isolated strains showed

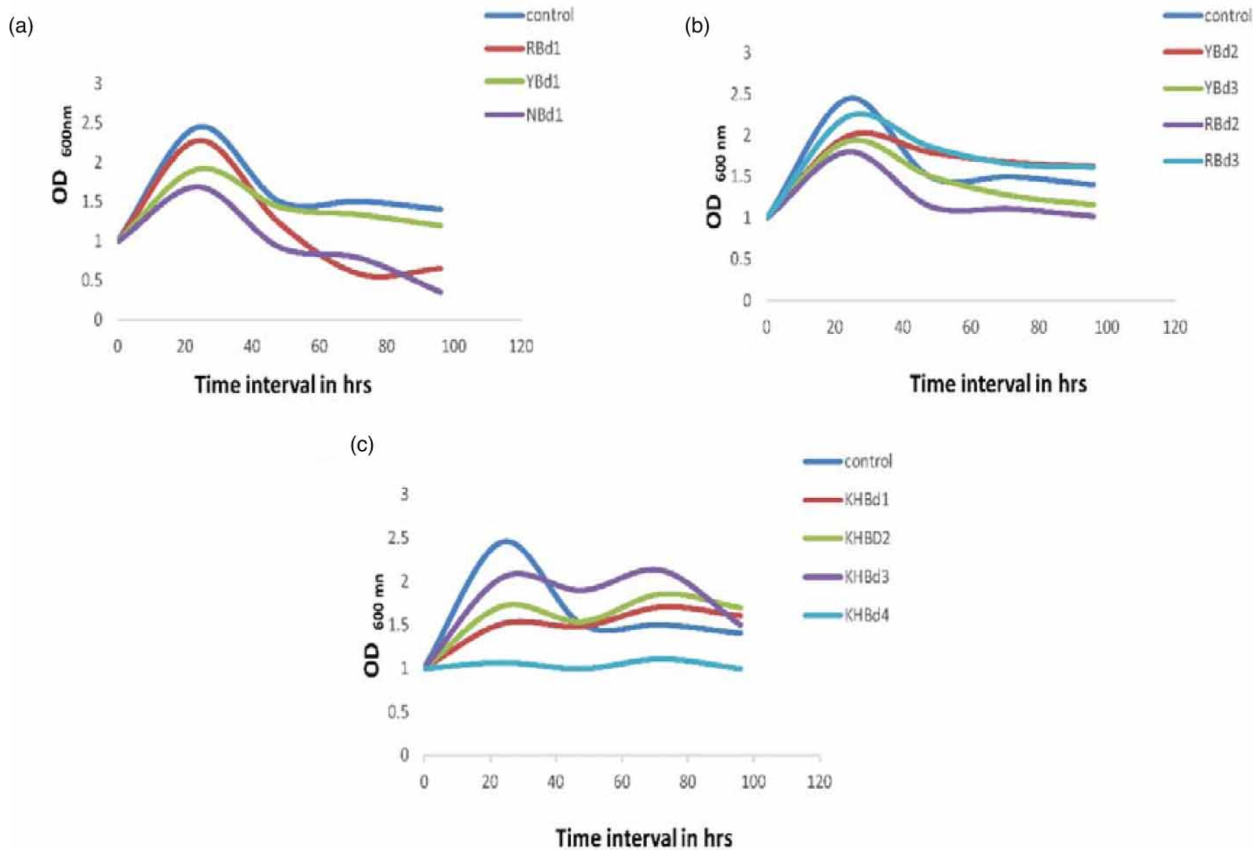

Figure 6 | Graphical representation of lytic activity of *Bdellovibrio* isolates against *Salmonella typhimurium*: (a) activity of NBd1, YBd1 and RBd1; (b) activity of YBd2, YBd3, RBd2 and RBd3; (c) activity of KHBd1, KHBd2, KHBd3 and KHB4.

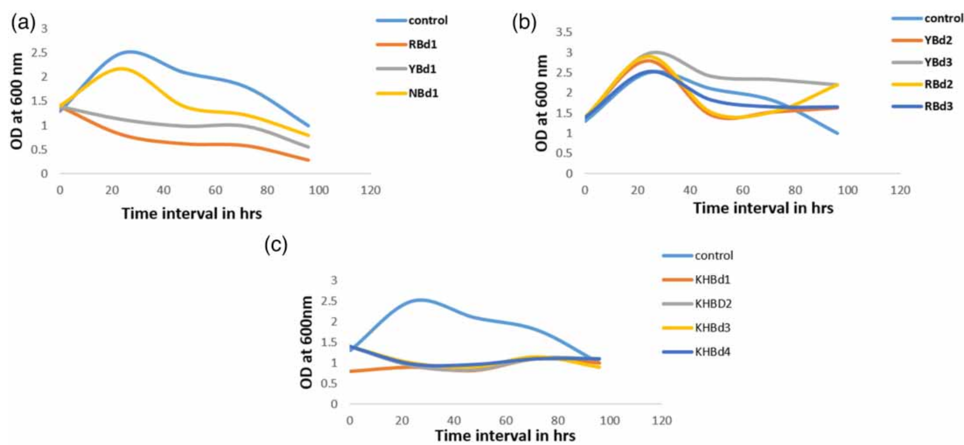

Figure 7 | Graphical representation of lytic activity of *Bdellovibrio* isolates against *V. cholerae*: (a) activity of NBd1, YBd1 and RBd1; (b) activity of YBd2, YBd3, RBd2 and RBd3; (c) activity of KHBd1, KHBd2, KHBd3 and KHB4.

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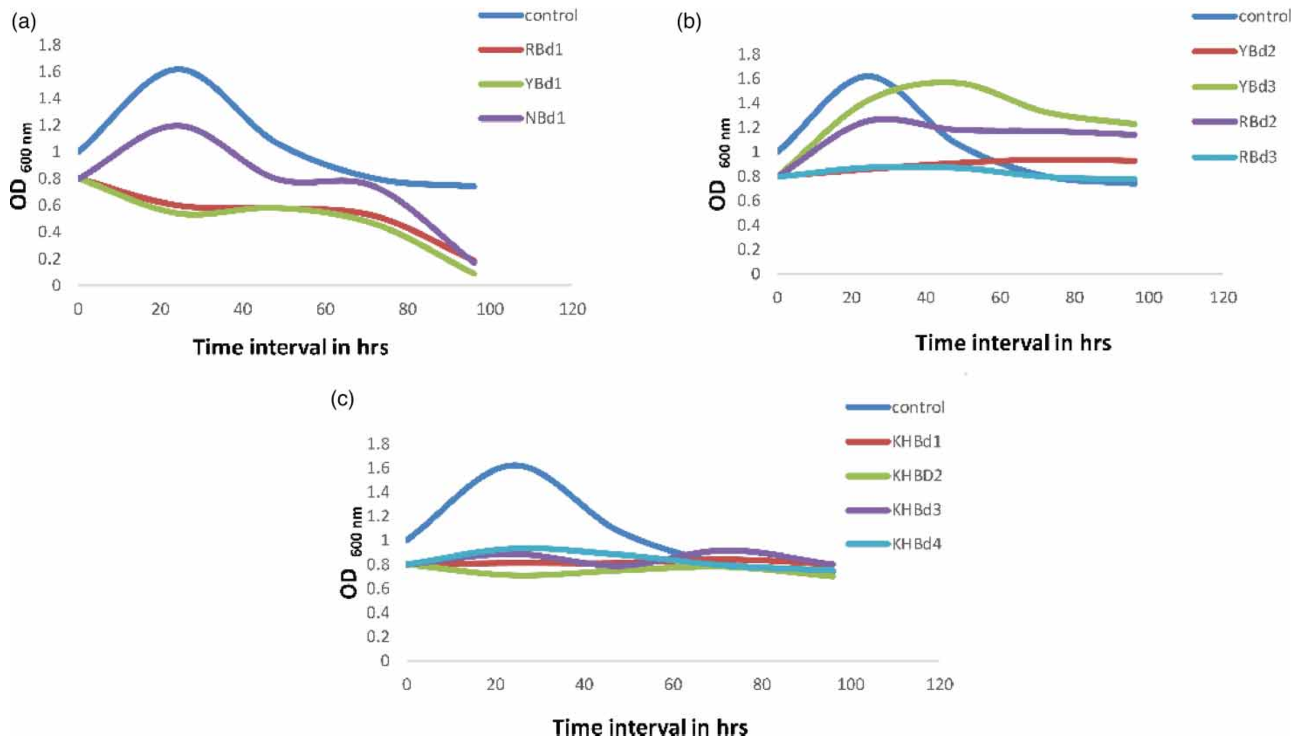

Figure 8 | Graphical representation of lytic activity of *Bdellovibrio* isolates against *Pseudomonas aeruginosa* ADW44: (a) activity of NBd1, YBd1 and RBd1; (b) activity of YBd2, YBd3, RBd2 and RBd3; (c) activity of KHBd1, KHBd2, KHBd3 and KHBd4.

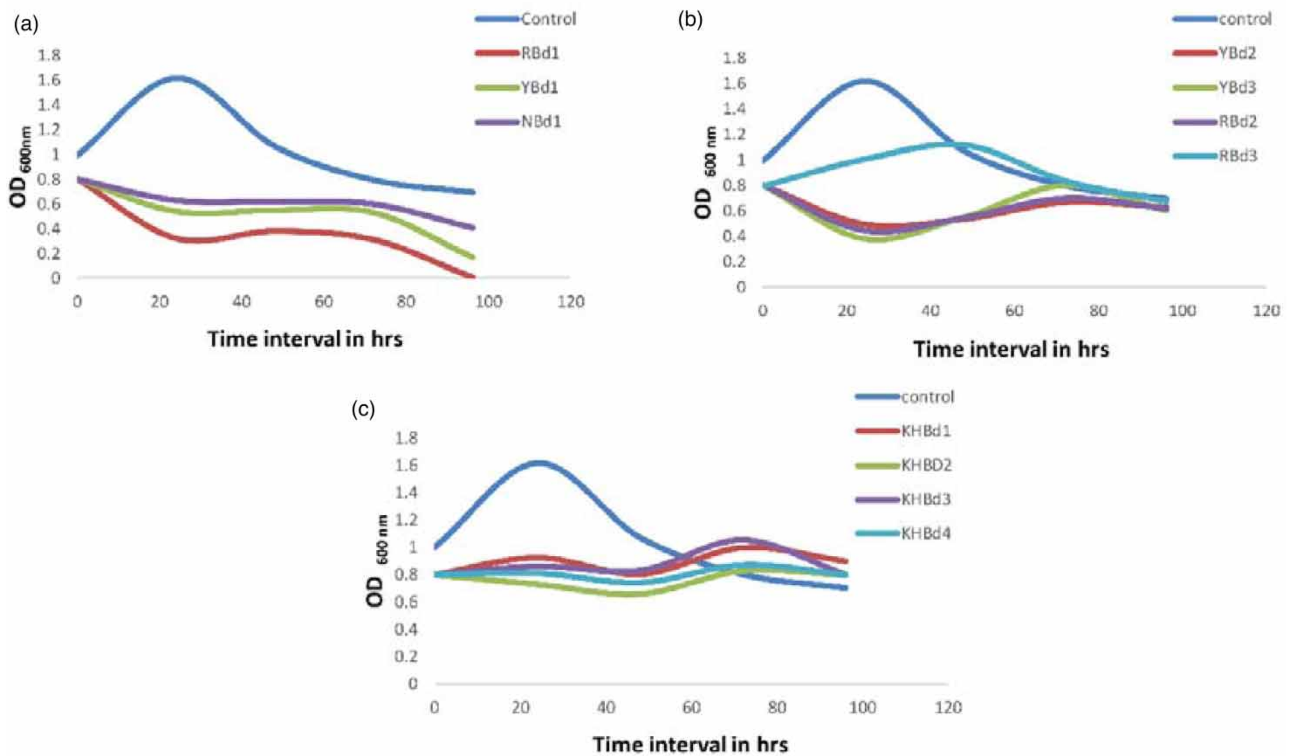

Figure 9 | Graphical representation of lytic activity of *Bdellovibrio* isolates against *Pseudomonas aeruginosa 27853*: (a) activity of NBd1, YBd1 and RBd1; (b) activity of YBd2, YBd3, RBd2 and RBd3; (c) KHBd1, KHBd2, KHBd3 and KHBd4.

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different inhibition rates toward the tested organisms after 24 h of incubation and it was measured based on the reduction in OD_{600} at specific time intervals. The predation efficiency was observed to be preferential because each *Bdellovibrio* strain showed different predatory activities against the tested organism.

Figure 8 presents the predation of all 11 *Bdellovibrio* isolates against *P. aeruginosa* ADW44. Reduction in OD (OD_{600nm}) after 24 h of incubation was observed when the *P. aeruginosa* ADW44 cultures were mixed with RBd1, YBd1, NBd1 isolates. The remaining *Bdellovibrio* strains YBd2, YBd3, RBd2, RBd3, KHBd1, KHBd2, KHBd3 and KHBd4 did not show much reduction in OD $_{600nm}$ as compared with RBd1, YBd1, NBd1. Similar results were exhibited by these *Bdellovibrio* strains against other three tested organisms such as *S. typhimurium, V. cholerae* and *P. aeruginosa* 27853. But interestingly, the reduction in OD by YBd3 strain against *V. cholerae* did not match with the control. Overall, in the present study, three *Bdellovibrio* isolates namely RBd1, Ybd1 and NBd1 showed effective predation against all the tested organisms.

DISCUSSION

Antibiotic resistance in bacteria is a major concern in both national and international scenarios. Since the discovery of *Bdellovibrio* in 1962 (Stolp & Starr 1963), they are recognized as the predator species capable to prey Gram-negative bacteria which may impart a role in regulating microbial communities in environment (Caulton & Lovering 2020). The objective of the present study was to evaluate the lytic activity of *Bdellovibrio* isolated from untreated hospital wastewater against selected bacterial pathogens.

A total of eleven *Bdellovibrio* isolates were obtained from 30 untreated wastewater samples. Primary isolation of *Bdellovibrio* was performed using *P. aeruginosa* ADW44 as a prey organism. In another study, a total of 14 *Bdellovibrio* strains were obtained from the cultured fishponds using *Aeromonas hydrophila* J-1 as host (Chu & Zhu 2010). Lytic plaques were visible after 3–5 days of incubation and increased in size on further incubation. This phenotypic observation suggested that the predators were motile in nature within the soft agar. This observation clearly differentiates them from the phages (Feng *et al.* 2016). All 11 isolates of *B. stolpii* were obtained using the double-layer agar technique. This study supports the use of a double-layer agar plating technique and the use of dilute nutrient broth as an effective method for the isolation of *Bdellovibrio* strains from wastewater.

All the isolated *Bdellovibrios* in the present study were confirmed as *B. stolpii* by PCR. To the best of our knowledge, this is the first study to report the isolation of *B. stolpii* from hospital wastewater samples in India. *Bdellovibrios* have been previously isolated from soil, marine and rhizosphere, fresh water, brackish water, seawater, sewage water and animal intestine indicating their diverse prevalence in a terrestrial and aquatic environment but predominantly inhabiting *B. bacteriovorus* species (Medina *et al.* 2008).

The availability of genetic data to characterize *Bdellovibrio* is limited where most of the sequences in databases are 16S rDNA sequences used for phylogenetic treeing. In the present study, all 11 isolates were screened for 16S rDNA coding region using specific primers by providing desired amplicon size. Species-specific primers were used for confirmation of *Bdellovibrio* species. In the present study, the *hit* locus was successfully amplified in *Bdellovibrio* with an amplicon of two different sizes (Figure 4) which suggests that the isolates are different strains. Similar findings were obtained in another study by Oyedara *et al.* (2016). The presence of one genetic locus, *hit* is responsible for the transition from the attack phase to the growth phase in *Bdellovibrio* and the presence of this gene in isolates from our study strongly correlates with the study by Cotter & Thomashow (1992). The conversion of host-dependent strains of *Bdellovibrio* to saprophytic strains which can grow on heat-killed host bacteria is influenced by the mutations in the *hit* locus (Jurkevitch *et al.* 2000).

The activity of *Bdellovibrio* isolates was evaluated against four different clinical prey species such as *P. aeruginosa* ADW44, *P. aeruginosa 27853, V. cholerae* and *S. typhimurium* in dilute nutrient broth. In the present study, three *Bdellovibrio* isolates (RBd1, NBd1 and YBd1) exhibited good predatory behavior against the tested clinical isolates. Isolated strains of *B. stolpii* in the present study showed different killing rates among the tested clinical isolates. The predatory efficacy of YBd3 strain against *V. cholerae* in the present study is quite deviated when compared with the control. Similar findings were obtained in another study conducted by Rogosky *et al.* (2006), where differential predation by *B. bacteriovorus* 109J was observed when the predator cells were mixed and incubated with two types of prey cells. The observations drawn in the present study are also supported by other study findings where *B. bacteriovorus* UP readily preyed upon Gram-negative bacteria but not Gram-positive bacteria including the isolates that originated from the same sources (Feng *et al.* 2016). This finding has led to the conclusion that the selection of the appropriate predator for clinical purposes would require larger and more in-depth studies on predation to overcome predation resistance.

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The inability of other remaining *Bdellovibrio* species to prey on other host cells could also be due to its structural features, which inhibit attachment, penetration or replication or may be due to the escape strategy of the host cell, which is not studied in the present study. Recent studies demonstrated applications of *Bdellovibrio* strains in biological wastewater treatment for reducing the burden of Gram-negative bacteria (Wu *et al.* 2019; Jafarian *et al.* 2020).

Bdellovibrio is able to prey on different clinical strains of a variety of beta-lactamases producing MDR Gram-negative bacteria regardless of their antibiotic resistance (Markelova 2010). In the present study, Figure 8 shows that *P. aeruginosa* ADW44, despite being the MDR, strong biofilm-forming bacteria was more susceptible to predation by *B. stolpii* than other prey cells used. YBd2, YBd3 and RBd2 effectively reduced the growth of the prey organism at the moment between 20 and 40 h. The obligatory parasitic lifestyle and predatory behavior make a way for their extensive use as biocontrol agents and an alternative to antibiotics in horticulture, aquaculture, livestock farming, food processing and medicine (Pérez *et al.* 2020). In the present study, selected clinical isolates of *P. aeruginosa, S. typhimurium and V. cholerae* were tested for predation. Studies also showed that *Bdellovibrio* is capable of preying on various other strains belonging to *Acinetobacter, Escherichia, Bordetella, Aeromonas, Burkholderia, Pseudomonas, Citrobacter, Enterobacter, Klebsiella, Listonella, Morganella, Proteus, Vibrio, Salmonella, Yersinia, Shigella and Serratia* genera (Bonfiglio *et al.* 2020). The ability of *Bdellovibrio* to lyse the MDR pathogenic organism *P. aeruginosa* in the current study indicates their potential applications in various fields.

One unique characteristic about *Bdellovibrio* that distinguish it from bacteriophage is its ability to invade the biofilm, suggesting it is better suited than phage to environments with multiple preys irrespective of their antibiotic resistance. The predatory efficacy of bacteriophages and *Bdellovibrio* comparison has not been carried out in the present study. Due to the predatory behavior of *Bdellovibrio* may replace the use of antibiotics in livestock feed which will eventually help to control antimicrobial resistance in human pathogens. *Bdellovibrio* as a strategy to combat antibiotic resistance gene transfer in wastewater treatment plants may help them to use as a novel technique to overcome horizontal gene transfer from clinical isolates to the environment which is yet to be addressed fully. *Bdellovibrio* may maintain its ability to reduce bacterial loads and preformed biofilms in clinical settings, as well as the environment. Further studies may prove their ability to target biofilm formation among various bacteria. Higher lytic activity toward *Pseudomonas* strains shows that these pathogens are more susceptible to predation by *B. stolpii*. The present study gives a predatory spectrum of *B. stolpii* against biofilm-forming *P. aeruginosa* ADW44 a wound isolate and underlines its potential use as a biocontrol agent which may prevent delayed wound healing.

CONCLUSIONS

Bdellovibrio strains isolated in the present study demonstrated the capability to prey upon selected Gram-negative pathogens such as *P. aeruginosa, V. cholerae* and *S. typhimurium*. The method developed in this study will allow a more rigorous assessment of the potential use of *Bdellovibrio* as a biocontrol agent versus biofilms. The differences in the prey range and amplification of *hit* locus of the isolates observed support the presence of heterogeneous groups of *Bdellovibrio* species in this region. This suggests further characterization and classification of *Bdellovibrio* into different subgroups by sequencing. More interestingly, predatory bacterium having multiple host ranges will have a great medical application. Studying their non-toxicity and other non-pathogenic characteristics on human cell lines may highlight their potential application as biocontrol agents to fight the infection in a broad approach as an antibiotic. However, multifaceted research studies are required to prove the advantages of the predatory bacterium to put into regular use in treating MDR infections. Due to the limited research on *Bdellovibrio* in India, this work is expected to form a base for further research on them with the potential biotechnological applications as the ultimate goal.

ACKNOWLEDGEMENTS

The authors are grateful to acknowledge Nitte University Faculty Research Grant [Sanction Order No: N/RG/NUFR1/NUCSER/2020/04 for providing financial support to D.M. The facilities provided for the study in Nitte University Center for Science Education and Research, Nitte (Deemed to be University) are gratefully acknowledged.

DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

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CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 1 June 2022; accepted in revised form 12 January 2023. Available online 23 January 2023

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