



Antagonistic activity of *Exiguobacterium indicum* LIS01 isolated from sediment

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ABSTRACT

The bacterial resistance over antibiotics has gained significant concern worldwide for the last few decades. This study aimed to isolate and characterize antibiotic producing bacteria from different water sediment sources in Bangladesh. A total of 9 samples were collected from three different sediment sources from Gopalganj, Bangladesh. Bacterial isolates grown in nutrient agar with cycloheximide were tested for antagonistic activity following disc-diffusion assay. Based on preliminary screening, three isolates namely LIS01, LIS02 and LIS03 showed antibacterial activity against six clinical isolates: *E. coli*, *Salmonella* sp., *Shigella* sp., *Klebsiella pneumoniae*, *Streptococcus* sp., and *Staphylococcus* sp. The isolate LIS01 with the highest ($P < 0.05$) zone of inhibition was characterized using 16S rRNA gene sequencing. According to phylogenetic analysis, the isolate was identified as *Exiguobacterium indicum* with more than 99.7% sequence similarity to previously characterized strains. The results of our study are very promising considering the antibiotic resistant bacteria and could be helpful in treating diseases associated with drug failure caused.

INTRODUCTION

The emergence of drug resistance in clinical isolates causes severe health alarm globally. In developing countries with poor health and hygiene, the problem becomes worse day by day [1,2]. In addition, environmental pollution, mostly by industrial and medical waste generates super bugs through horizontal transfer of antibiotic resistant genes from pathogenic microbes into non-pathogenic isolates [3,4]. This cycle continues and gives rise to numerous superbugs, making the treatment process difficult and complicated. In Bangladesh, most recent studies revealed alarming drug resistant patterns in clinical isolates where bacteria gained complete resistance against third generation antibiotics (ceftriaxone,

cephalosporin and ciprofloxacin) [4–8]. Considering the side effects, environmental impacts, and sustainability, finding an alternative source of antibiotics has still remained the utmost priority in biological science and natural product research.

Environment is considered as the natural reservoir of a vast range of antagonistic bacteria with therapeutic applications [9–11]. Among the environmental bacteria, *Streptomyces*, *Bacillus* and *Pseudomonas* species are the most important bacteria reported to produce broad spectrum antibiotics having agricultural and medical applications [4,12–14]. Although previous studies demonstrated the antagonistic activity of these isolates against drug resistant clinical isolates [15–20], however re-emergence of virulent strains and ever

changing counter mechanisms of bacteria against drugs compelling researchers to find new antagonistic bacteria with potent anti-microbial activity. Bangladesh has a diverse land area, ocean, rivers, hills and mangrove forest that are reported as the most potent sources of antagonistic bacteria [21–23]. Especially the soil and sediment bacteria due to their diversity and distribution produce a wide range of bioactive metabolites and antimicrobial proteins [24–26]. In this backdrop, the present research aimed to characterize new, safe, eco-friendly, and potent antagonistic bacteria from the river sediment of Bangladesh and evaluate the effects against a broad range of clinical pathogens.

MATERIALS AND METHODS

Collection of bacterial isolates

Bacterial samples were collected from three different places of Gopalganj (23.0130° N, 89.8224° E), Bangladesh. The sampling points are Madhumoti river (latitude: 22° 52' 47" N, longitude: 89° 54' 45" E), Chandar Beel (23.2632° N, 89.9066° E) and Borni baor (22.9602° N, 89.8523° E). A total of 9 sediment samples (n = 3) were collected from at least 15 cm of depth with a sterile inoculating spoon during March and April 2019, placed in sterile plastic zip-lock bag and transported immediately (< 1 h) to the Biotechnology and Genetic Engineering-laboratory of the Bangabandhu Sheikh Mujibur Rahman Science and Technology University, Bangladesh, by maintaining cold chain with gel ice. Then, the samples were air dried according to methods described by Hossain and Rahman [23].

Culture conditions and biochemical characterization

For the isolation of bacteria, 10- fold serial dilution method was performed for 9 samples with phosphate buffered saline (PBS, pH 7.2). Sample from each dilution (10^4 - 10^7) was then streaked on a nutrient agar (NA) plate amended with cycloheximide (100 mg/ml) to prevent fungal growth and incubated at 37°C for 24 h [23]. Isolated colonies were then sub-cultured into NA plate and incubated overnight at 37°C [27]. Primary biochemical characterization was performed according to Bergey's manual for the identification of bacteria [28]. Further biochemical characterization was performed following methods for identification of *Bacillus* and *Exiguobacterium* species [29,30].

Evaluation of antagonistic activity

The antagonistic activity of bacterial isolates was measured by cross streak method described by Ran et al., 2012 [31]. A pure culture of each isolate was first grown on the nutrient broth (NB) and incubated for 24 h at 37°C. From the NB broth, 50 ml of culture was poured in 6 mm circular Whatman's filter paper, dried at room temperature, followed by disc-preparation using a punching machine [26]. Six clinical isolates namely *E. coli*, *Salmonella* sp., *Shigella* sp., *Klebsiella pneumoniae*, *Streptococcus* sp., and *Staphylococcus* sp. collected from Gopalganj medical college and hospital were used to evaluate the antagonistic activity of cultured isolates. The disc-diffusion assay was performed following methods described by Ran et al., 2020. These clinical isolates were grown on nutrient broth (NA) culture and incubated overnight at 37 °C. Then, 50 ml of bacterial culture was grown in the nutrient agar (NA) by spread plate method [26]. Then previously prepared filter paper discs of bacterial isolates were carefully placed onto the culture plate skeptically under the biological safety cabinet. In the present study, we analyzed 12 h, 18 h, and 24 h of bacterial culture for the inhibition assay. After 24 h of incubation, the zone of inhibition was measured according to the method described earlier [26]. Each experiment was performed as triplicate.

Characterization of potential antagonistic bacteria

The isolate with highest antagonistic activity was selected for further study. It was sub-cultured again on nutrient broth for molecular characterization using 16S rRNA sequencing. The overnight broth culture was used for bacterial genomic DNA extraction using Genomic DNA Mini Prep Kit (Bio Basic Inc., Ontario, Canada) according to manufacturer's instructions. The extracted DNA was checked for quality and quantity in 1% agarose gel using lambda (k) DNA marker and Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA) as a ratio of DNA-protein absorbance. The DNA was then stored at -20°C until further use.

PCR amplification of bacteria

The PCR was performed in 25 µl master reaction mixtures containing template 1.5 µl DNA (genomic DNA of bacteria), 2.5 µl of 25 mM MgCl₂, 2.5 µl of 10x colorless reaction buffer, 1.0 µl concentration of

deoxynucleotide triphosphate (dNTP), 1 µl of each of universal forward (27 F, 5'- AGA GTT TGA TCM TGG CTC AG -3') and reverse primer (1391 R, 5'- GGT TAC CTT GTT ACG ACT T -3') for bacteria, 0.5µl Taq DNA polymerase and 15 µL of nuclease-free water. A total of 35 cycles of amplification reactions were carried out in a MultiGene gradient thermal cycler (Labnet International Inc., USA) under following conditions; an initial denaturation step at 95°C for 5 min followed by a second denaturation step at 95°C for 40 sec, annealing for 1.0 min at 55°C, an elongation at 72°C for 2.0 min, and a final extension step of 72°C for 10 min [32].

Sequencing and analysis of 16s rDNA

The PCR product was purified using the PureLink PCR purification kit (ThermoFisher Scientific, USA) according to the manufacturer's guidelines. The purified PCR product was then sent to Invent Technologies Limited, Dhaka, for 16s rDNA sequencing. The sequences were then edited and assembled in Geneious software (vR11.1) [33] and before further phylogenetic analysis using MEGA 7.0 [34]. After primary nucleotide BLAST (BLASTn) in NCBI, multiple sequence alignment was performed as ClustalW in MEGA 7.0. The phylogenetic tree was constructed using neighbour-joining method with 1000 bootstrap replicates, taking *Streptomyces* sp. as the out-group strain. The nucleotide sequence of the bacteria is currently available at NCBI GenBank database under the accession number of MT742616.1.

Statistical analysis

One-way ANOVA with Tukey's post hoc test was applied to compare the mean values of antagonistic activity for three isolates (LIS01, LIS02 and LIS03). Alpha level of 0.05 was considered as statistically significant.

RESULTS

Isolation of bacteria

Based on morphological and biochemical tests (Table 1), three isolates primarily identified as Bacilli. However, isolate LIS01 showed differences in biochemical characteristics compared to LIS02 and LIS03, indicating a different bacteria strain.

Table 1. Biochemical characterization of isolates

Characteristics	LIS01	LIS02	LIS03
Grams's staining	+	+	+
Catalase	+	+	+
Oxidase	-	-	-
Gelatinase	-	+	+
Lipase	-	-	-
Strach	-	+	+
Citrate	+	+	+
Methyl-red	-	+	+
Nitrate reduction	+	+	+
Voges-Proskauer	+	+	+
Acid production (glucose)	-	+	+

Antagonistic activity of *E. indicum* LSA01

In primary screening, the isolate *E. indicum* LSA01 showed significantly higher ($P<0.05$) antagonistic activity compared to other two isolates (LIS02 and LIS03) (Figure 1). The *E. indicum* LSA01 showed potential inhibitory effects against all the six tested clinical isolates in the *in vitro* plate assay. Study results revealed that the overnight culture (24 h) was most effective ($P<0.05$) against tested isolates, followed by 18 h and 12 h, respectively. The highest zone of inhibition with *E. indicum* LAS01 was 16.3 mm for *E. coli*, followed by 15.3 mm for *Shigella* sp., 12.3 mm for *Salmonella* sp., and *Streptococcus* sp., and 10.7 mm for *K. pneumoniae*, respectively (Table 2).

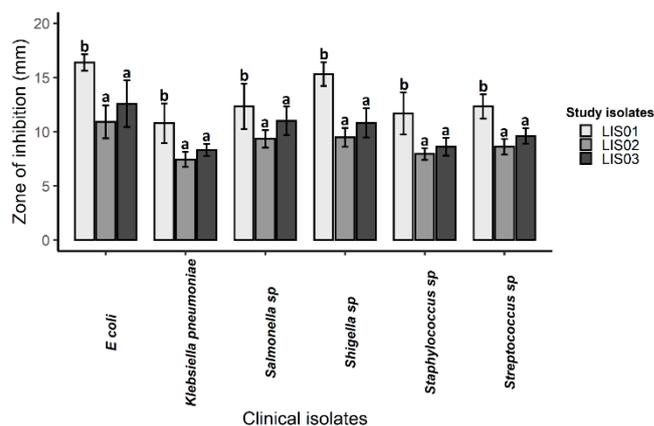


Figure 1. Inhibitory activity of present study isolates (LIS01, LIS02 and LIS03) against six clinical bacteria. Bar with different superscripts indicates significantly different mean values at 0.05 level of significance.

Table 2. Antagonistic activity (mean \pm SE) of *E. indicum* LSA01 against six clinical isolates

Isolates	Zone of inhibition (mm)		
	12 h	18 h	24 h
<i>E. coli</i>	11.3 \pm 0.6 ^a	11.7 \pm 1.2 ^a	16.3 \pm 1.2 ^b
<i>Shigella</i> sp.	8.0 \pm 0.6 ^a	10.7 \pm 1.2 ^a	15.3 \pm 1.2 ^b
<i>Salmonella</i> sp.	8.3 \pm 0.3 ^a	9.0 \pm 0.3 ^a	12.3 \pm 0.9 ^b
<i>Streptococcus</i> sp.	8.3 \pm 1.2 ^a	10.7 \pm 0.9 ^b	12.3 \pm 0.9 ^c
<i>Staphylococcus</i> sp.	11.7 \pm 1.5 ^a	10.3 \pm 0.9 ^a	11.7 \pm 0.6 ^a
<i>Klebsiella pneumoniae</i>	8.7 \pm 0.9 ^a	9.0 \pm 0.3 ^a	10.7 \pm 0.9 ^b

Row with different superscripts indicates significantly different mean values at 0.05 level of significance.

Identification of bacteria and phylogenetic analysis

The modified tests for the classification of *Exiguobacterium* genus [29] confirmed the highest antibiotic producing isolate LIS01 as *E. indicum*. The NCBI nucleotide BLAST (BLASTn) and phylogenetic tree analysis using 16S rRNA data confirmed the species and relationship of LIS01 to previously identified *E. indicum* (Figure 2). Neighbour-joining tree revealed close clustering of *E. indicum* LSA01 with other previously identified *E. indicum* including strain isolated from lake, river, and marine water in India. The study isolates also upheld close relationship to *Bacillus* species, however distant to *Streptomyces* species, as shown in phylogenetic tree (Figure 2).

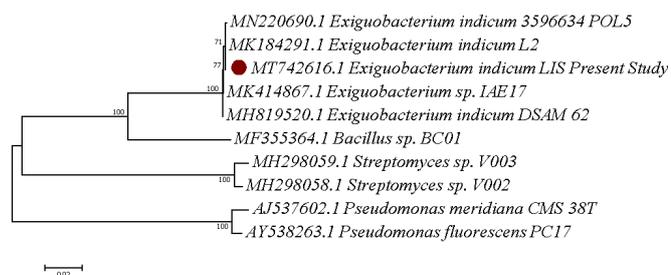


Figure 2. Phylogenetic tree showing the position and relationship of present study *E. indicum* LIS01 isolate to other strains characterized earlier. The tree was constructed using neighbour-joining method in MEGA 7. *Streptomyces* sp., and *Pseudomonas* sp., were considered as out group bacteria in the tree.

DISCUSSION

Emergence of antibiotic resistant bacteria compelled scientists to find new and effective drugs to prevent and treat many diseases of human. Compared to other sources, biocontrol agents are effective, safe and eco-friendly, and therefore used in novel drug developments since the discovery of penicillin [35].

Nature represents the most significant resource for the biologically active compounds that can be used as antibiotics to fight against bacterial diseases [36]. In this study, we have identified an *Exiguobacterium* species that found to have potent inhibitory activity against six MDR clinical isolates. The isolate was confirmed as *E. indicum* by biochemical and 16S rRNA based sequencing. The 16S rRNA method has been considered as gold standard for the identification of bacterial species [37,38]. Considering the drug resistant patterns in clinical isolates, the *E. indicum* species could be used as potential antibiotic candidate for treating diseases associated with these six bacteria.

The isolates were screened for antibiotics production through disc diffusion in the *in vitro* plate assay. This is an agreement with some previous literatures that used the same methods to screen prospective antibiotic producing isolates [26,39,40]. In present study, *E. indicum* strongly inhibit *E. coli* and *Shigella* spp., indicating its higher efficacies against food-borne pathogens. However, the activity was significantly lower for *Staphylococcus* and *Klebsiella*, two most drug resistant clinical bacteria characterized from urinary tract infected (UTI) patients in Bangladesh [41–43]. These results are inconsistent with some previous studies where the higher inhibitory activity of *Streptomyces* and *Bacillus* species was recorded against *E. coli* compared to *Streptococcus*, *Staphylococcus* and *Klebsiella* species [17,44,45]. In recent studies, complete drug resistant *Staphylococcus* and *Klebsiella* isolates have been identified from patients with UTI revealing the severity of drug incompetence in Bangladesh [41].

The phylogenetic data of *E. indicum* revealed a close relationship of present study isolate with other antagonistic *Exiguobacterium* species isolated from soil, water and sediments [41,46–47]. Out of these close neighbours, *E. indicum* POL5 had highest similarity score to present study isolate that is reported as potential inhibitor of pathogenic bacteria. To compare with other potential antagonistic strains, we found that present study isolate was distinctly different from *Bacillus* sp. BC01 and *Streptomyces* species. Therefore, it is quite clear that, present study bacteria are a new strain of *E. indicum* with promising antagonistic activity against clinical isolates.

After investigating the results, the present study strain could be used as potential antibiotic candidate after purification and necessary strain development processes. However, considering the limited resources and financial constraints, those steps were beyond our

study objectives. In addition, the number of clinical isolates (six species) tested is not big enough to make a conclusion about the broad-spectrum antagonistic activity of present study bacteria. Therefore, inhibitory activity should be measured with big numbers and greater volume of clinical samples. Further genome sequencing and functional annotations are required to identify the candidate genes associated with antagonistic activity.

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None

AUTHOR CONTRIBUTIONS

Asura Khanam Lisa designed the study, performed laboratory assays, analyzed the data and prepared the draft manuscript. Most. Sumya Alam and Fabia Hannan Mone collected samples and performed inhibitory assays. Abdullah-Al-Jubayer analysed the data and reviewed the manuscript. All authors approved the final manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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