Influence of multi-species biofilm formed in vitro from different environmental samples on the drug-resistance traits of resident bacteria

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Present study was designed to investigate whether the formation of multi-species biofilm impart any effect on the enhancement of the antibiotic resistance of the planktonic cells residing in the general nutrient enriched environments. In this regard, surface water of pond, mud from agricultural field, pharmaceutical waste waters, municipal waste water, hospital waste water and the domestic waste samples were collected and were induced to form an in vitro multi-species biofilm. All samples were found to contain huge array of pathogenic bacteria. The pathogenic isolates both from the bulk samples and their corresponding biofilms were subjected to antibiogram assay using 13 commonly used antibiotics. An extended frequency of drug resistance was observed in case of biofilm isolates. The findings of present study revealed that a number of bacterial isolates which showed sensitivity towards antibiotics, acquired drug-resistance when they were isolated from biofilms.

Key words: Multi-species biofilm, drug resistance, surface water, waste water

Introduction

Antibiotic resistance has been a global interference in disease medication principally shaded by the pathogenic microorganisms, especially in the developing countries with high density of population under the line of poverty as in Bangladesh.¹⁻⁹ Unethical usage of non-prescribed antibiotics and clinically unsafe practice of self-medication led to the development of the unsolicited resistance by the infecting bacteria.²⁻⁵,¹⁰ Several studies in Bangladesh found extended numbers of drug resistant isolates including the multi-drug resistant (MDR) ones in different food and water samples, analytical of their possible propagation into the natural environments.⁵,¹¹⁻¹⁵ The mechanism of bacterial drug-resistance is usually known to be based on the production of inactivating enzymes, change in the drug-target sites, and the exclusion of the antibiotic from the target site.¹⁶ Even the efficiency of the prescribed antibiotic may be threatened by genetic mutation and the possible spread of the drug-resistant genes through horizontal or vertical transmission, or through the extra-chromosomal plasmid genes, and finally by the transposons.²⁻¹⁷,¹⁸

Majority of bacteria in natural habitats, especially in natural aquatic ecosystems are organized in biofilms, simply defined as microbial communities that are attached to a surface and embedded in a self-produced matrix composed of extracellular polymeric substances.¹⁹⁻²¹ Although single-species biofilms exist in a variety of infections and on the surface of medical implants, multi-species biofilms may also predominate in the natural environments.²¹⁻²³ The multi-species biofilms may indeed offer heightened opportunities for interactions such as horizontal gene transfer and co-metabolism in natural environments which may take part in the elevated drug resistance.

Most of the studies concerning drug resistance in the environmental samples have focused on the bulk sample and situation of individual bacteria isolated from that samples.⁵,¹¹⁻¹⁵ However, those studies did not focus the situation in case of biofilms, which is widely known to be the preferred pattern of life of many bacteria regarding bacterial survival in the adverse environment. High bacterial density and diversity are commonly observed in biofilms from wastewater systems as well as from surface water and drinking water distribution systems where antibiotic resistance may frequently spread.¹⁸,²⁴,²⁵ Considering all these facts, present study endeavored to estimate the possible occurrence of the enhanced drug resistance of bacterial isolates due to their participation in the formation of the multi-species biofilms from surface water and different waste water samples.

Materials and methods

Study period and sampling

The study was carried out at the Microbiology Laboratory, Stamford University Bangladesh from August 2016 to October 2016. Different nutrient rich aqueous samples such as surface water of pond, mud from agricultural land, municipal waste water, pharmaceutical waste water, domestic waste and hospital waste water were randomly collected in sterile PET bottle or jar from different locality of Dhaka city and transported to the laboratory at the earliest convenient. For the isolation and enumeration of

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pathogenic bacteria, 10 ml or g of each sample was homogenized in 90 ml normal saline and diluted up to 10⁻⁵ according to the standard guideline

**Estimation of total viable bacteria, Escherichia coli, Klebsiella spp., Staphylococcus spp. and Bacillus spp.**

From the dilutions 10⁻³ and 10⁻⁵, 0.1 ml of each sample was spread onto the nutrient agar (NA) media for the enumeration of total viable bacteria. Likewise, 0.1 ml of each sample from the raw suspension and dilution 10⁻² were introduced onto MacConkey agar, mannitol salt agar (MSA), mannitol egg yolk polymyxin (MYP) agar and cetrimide agar for the isolation of coliforms (Escherichia coli and Klebsiella spp.), Staphylococcus spp., Bacillus spp. and Pseudomonas spp., consecutively. All the plates were then incubated at 37 °C for 24 hours.

**Isolation of Salmonella spp., Shigella spp. and Vibrio spp.**

By considering the possible occurrence of viable but non-culturable (VBNC) cells, 10 ml of sample was transferred into 90 ml of selenite cysteine broth (SCB) and alkaline peptone water (APW) for the enrichment of Salmonella, Shigella, and Vibrio spp., respectively and incubated at 37 °C for 6 hours. After incubation, the samples were diluted up to 10⁻⁴ and then 0.1 ml of samples from each of the 10⁻² and 10⁻⁴ dilutions were spread onto Salmonella-Shigella (SS) agar and thiosulfate citrate bile salt sucrose (TCBS) agar for the isolation of Salmonella spp. & Shigella spp., and Vibrio spp., consecutively.

**Biochemical identification of the bacterial isolates**

Finally, all the isolates were biochemically examined for their identification following standard procedures as described earlier.

**In vitro biofilm formation and recovery of the planktonic cells**

On the other hand, 10 ml or g of each sample was introduced and homogenized in 90 ml Luria-Bertani (LB) broth soon after the arrival of the samples and then incubated at 37 °C with constant shaking at 150 rotations per minute (rpm) for three to seven days. Planktonic (free-living) bacteria residing in each sample was allowed to grow in the broth medium until nutrient was declined which was the selective pressure for biofilm formation. As a consequence, drop in planktonic bacterial cell number would result in the formation of biofilm. Macroscopic flocs of thin opaque layer suspended surrounding the surface of the medium anchoring flask was the indicative of biofilm formation.

The planktonic cells were further recovered from the biofilm by isolating them on the specific media. One loopful culture from the biofilm of each sample, which was collected very carefully from the surface of the broth medium, was inoculated onto MacConkey agar, MSA agar, MYP agar and cetrimide agar for the isolation of coliforms (Escherichia coli and Klebsiella spp.), Staphylococcus spp., Bacillus spp. and Pseudomonas spp., consecutively. Isolation of Salmonella spp., Shigella spp. and Vibrio spp. was carried out following the same procedure as described previously.

**Antibiotic susceptibility test of the isolates**

The standard agar-disc-diffusion method (Kirby Bauer technique) was used to examine the antibiotic susceptibility of the pathogenic bacteria isolated directly from the samples as well as from the biofilm formed from the same samples on Mueller-Hinton agar (Difco, Detroit, MI) with same set of antibiotics. Antibiotics used in this study included trimethoprim/sulfamethoxazole (25 µg), erythromycin (15 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), cefixime (5 µg), polymyxin B (300 units), imipenem (10 /µg), gentamicin (10 µg), nalidixic acid (30 µg) and azithromycin (15 µg).

**Results and discussion**

Unregulated disposal of industrial, hospital and domestic wastes containing organic substances along with toxic chemicals including antibiotics as well as pathogenic bacteria to the environment may play a huge role in the accumulation of bacterial drug-resistance. As stated elsewhere, most of the studies in Bangladesh concerning antibiotic resistance of bacteria and their distribution in the environment are based on the analysis of bulk water, food samples and patients’ derived isolates so far. To add a new dimension to this area of research, multi-species biofilms, which are commonly formed in the environment by the close interaction of bacteria, were analyzed in this study.

**Determination of the presence of pathogenic bacteria from the environmental samples tested**

All the samples were found to contain huge load of viable bacteria in a range of 2.3×10⁵ - 1×10⁸ cfu/ml or g (Table 1) as found previously from such environmental samples. Pathogenic bacteria were also recovered in significant quantities from all the samples, especially surface water sample contained all the pathogenic bacteria tested except Shigella spp. which was actually absent in all samples. Klebsiella spp., Staphylococcus spp., Pseudomonas spp. and Bacillus spp. were found to be present in all the samples in the average of 10³, 10⁴, 10⁵ and 10⁶ cfu/ml or g, consecutively (Table 1). E. coli was absent in pharmaceutical and hospital waste water samples but resided in the other four samples within the range of 2.5×10³ - 3.3×10⁴ cfu/ml or g. Salmonella spp. was isolated from surface water, municipal waste water and domestic waste samples with average load of 10⁴ cfu/ml or g (Table 1). Only surface water sample exhibited the presence of Vibrio spp (1.3×10³ cfu/ml). Interestingly, pathogenic bacteria which were isolated from the tested environmental samples were also recovered from the biofilms of the samples.

**Enhanced antibiotic resistance of the isolates recovered from biofilms**

In agreement with the previous studies, the result of antibiogram assay for all the samples apparently showed that
those bacterial isolates which were found to be sensitive against any of the antibiotics used, exhibited resistance against that antibiotic in almost all instances when they were isolated from biofilms (Tables 2-7). The only exception was gentamicin ((GEN, 10 µg), against which none of the isolates showed resistance whether they were from bulk samples or from biofilms (Tables 2-7). In cohort to the previous studies on surface water and wastes\textsuperscript{11,12,40,43}, present study recovered a significant number of multidrug resistant isolates. The frequency of MDR cases was exceedingly accelerated when the bacteria were isolated from biofilm. If the samples were more specifically assessed, the isolates from surface water sample which exhibited sensitivity against trimethoprim-sulfamethoxazole (TRI-SUL, 25 µg), erythromycin (ERY, 15 µg), ceftriaxone (CEF, (30 µg), ampicillin (AMP, 10 µg), polimyxin B (POL-B, 300 units) and nalidixic acid (NAL, 30 µg) gained resistance when they were allowed to grow in biofilm (Table 2). A few of the isolates were found to keep their sensitivity against some antibiotics. \textit{Klebsiella} spp. remained to be sensitive against tetracycline (TET, 30 µg) and cefixime (CFX, 5 µg) besides GEN. \textit{Pseudomonas} spp. retained sensitivity against CFX, imepenem (IPM, 10 µg) and azithromycin (AZI, 15 µg). The sensitivity of \textit{Vibrio} spp. remained to be unchanged against chloramphenicol (CHO, 30 µg) and ciprofloxacin (CIP, 5 µg). \textit{Salmonella} spp. retained sensitivity against TET (Table 2).

In the mud sample, all the sensitive isolates acquired resistance against TRI-SUL, CEF, CIP, AMP, TET, CHO, POL-B and NAL (Table 3). \textit{Klebsiella} spp. remained sensitive against CFX and AZI, whereas \textit{Pseudomonas} spp. stayed sensitive against IPM.

### Table 1: Isolation and quantification of pathogenic bacteria from different environmental samples.

| Sample          | TVB (cfu/ml or g) | E. coli (cfu/ml or g) | Klebsiella spp. (cfu/ml or g) | *Salmonella* spp. (cfu/ml or g) | *Pseudomonas* spp. (cfu/ml or g) | Bacillus spp. (cfu/ml or g) | *Vibrio* spp. (cfu/ml or g) | Staphylococcus spp. (cfu/ml or g) |
|-----------------|-------------------|-----------------------|------------------------------|----------------------------------|----------------------------------|-----------------------------|-------------------------------|---------------------------------|
| Surface water   | 1.2×10⁹           | 3.3×10⁴               | 3.4×10³                      | 3.0×10⁴                          | 3.0×10⁴                          | 2.3×10³                     | 1.3×10³                       | 3.0×10⁵                         |
| Mud             | 2.3×10⁶           | 2.5×10³               | 1.5×10³                      | 0                               | 2.0×10³                          | 2.9×10³                     | 0                             | 2.0×10⁴                         |
| Municipal waste water | 1.5×10⁶          | 2.8×10³               | 2.9×10³                      | 3.8×10⁴                          | 2.1×10³                          | 3.8×10³                     | 0                             | 2.2×10⁵                         |
| Pharmaceutical waste water | 3.3×10⁶       | 0                    | 2.8×10³                      | 0                               | 3.9×10³                          | 2.0×10³                     | 0                             | 1.1×10³                         |
| Hospital waste water | 2.7×10⁶          | 0                    | 1.4×10⁴                      | 0                               | 1.0×10³                          | 1.5×10³                     | 0                             | 3.1×10⁴                         |
| Domestic waste  | 4.0×10⁷           | 2.2×10⁴               | 4.3×10³                      | 2.2×10⁴                          | 2.2×10³                          | 2.0×10³                     | 0                             | 2.9×10⁵                         |

TVB- Total viable bacteria
The experiments were in triplicates. Average count (cfu/ml or g) from all samples have been shown here.
* Bacterial load after enrichment (Prior to enrichment, the recovery was nil).
Shigella spp. was absent in all samples.

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### Table 2: Antibiotics susceptibility pattern of the isolates from surface water

| Antibiotics Isolates | TRI-SUL | ERY | CEF | CIP | AMP | TET | CHO | CFX | POL-B | IPM | GEN | NAL | AZI |
|----------------------|---------|-----|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|
| Bulk sample          | E. coli | S   | S   | ND  | S   | R   | R   | S   | S     | ND  | S   | S   | S   |
| Klebsiella spp.      | S       | S   | ND  | R   | S   | S   | S   | S   | ND    | S   | S   | R   | S   |
| Salmonella spp.      | R       | S   | S   | S   | S   | S   | ND  | ND  | S     | S   | S   | ND  | S   |
| Pseudomonas spp.     | S       | ND  | S   | S   | S   | S   | S   | ND  | S     | S   | S   | R   | S   |
| Bacillus spp.        | S       | S   | ND  | S   | S   | S   | ND  | S   | S     | S   | S   | ND  | S   |
| Vibrio spp.          | R       | S   | ND  | S   | R   | R   | S   | R   | S     | ND  | S   | ND  | S   |
| Staphylococcus spp.  | S       | S   | S   | S   | S   | S   | ND  | R   | ND    | S   | ND  | S   | S   |

Biofilm

| E. coli             | R       | R   | ND  | R   | R   | R   | R   | ND  | R     | S   | R   | R   |
| Klebsiella spp.     | R       | R   | ND  | R   | S   | R   | S   | ND  | R     | S   | R   | R   |
| Salmonella spp.     | R       | R   | R   | R   | R   | S   | R   | ND  | R     | ND  | S   | R   | ND  |
| Pseudomonas spp.    | R       | ND  | R   | R   | R   | S   | ND  | S   | S     | S   | R   | S   |
| Bacillus spp.       | R       | R   | ND  | R   | R   | ND  | R   | R   | S     | ND  | R   | S   | ND  |
| Vibrio spp.         | R       | R   | ND  | R   | R   | S   | R   | R   | S     | ND  | R   | S   | ND  |
| Staphylococcus spp. | R       | R   | R   | R   | R   | R   | R   | ND  | R     | ND  | S   | ND  | R   |

ND: Not done; R: Resistant; S: Sensitive
TRI-SUL= Trimethoprim/sulfamethoxazole (25 µg); ERY= Erythromycin (15 µg); CEF= Ceftriaxone (30 µg); CIP= Ciprofloxacin (5 µg); AMP= Ampicillin (10 µg), TET= Tetracycline (30 µg), CHO= Chloramphenicol (30 µg), CFX= Cefixime (5 µg), POL-B= Polymyxin B (300 units), IPM= Imepenem (10 µg), GEN= Gentamicin (10 µg), NAL= Nalidixic acid (30 µg); AZI= Azithromycin (15 µg).
along with these two antibiotics. The sensitivity of *Staphylococcus* spp. persisted against ERY (Table 3). In the municipal waste sample, all the sensitive isolates were found to be resistant against AMP, CHO, POL-B and NAL afterwards (Table 4). Almost all the isolates with one exception gained resistance against TRI-SUL, CIP, TET, ERY, and CEF (Table 4). Interestingly, the sensitive isolates retained their sensitivity against CFX. Two of the isolates remained sensitive each against IPM and AZI (Table 4).

In the pharmaceutical waste sample, all the sensitive isolates showed resistance against TRI-SUL, CEF, CIP, AMP, TET, CHO, POL-B and NAL when they were isolated from biofilm (Table 5). *Bacillus* spp. and *Staphylococcus* spp. which were found to be sensitive towards almost all antibiotics, acquired resistance against almost all of them afterwards and so was observed in other samples also (Tables 2-7). *Pseudomonas* spp. retained their sensitivity against CFX, IPM and AZI (Table 5). In hospital waste water samples, the sensitive isolates attained resistance against TRI-SUL, ERY, CEF, CIP, AMP, TET, CHO, POL-B and NAL (Table 6). As found in the other two samples, the sensitivity of the isolates remained to be unchanged against CFX. In addition to this, *Pseudomonas* spp. retained their sensitivity against IPM (Table 6). In the domestic waste sample, against AMP and POL-B, all the sensitive isolates gained resistance afterwards (Table 7). Almost similar findings were observed against TRI-SUL, ERY, CEF, CIP, TET, CHO and NAL with the retention of a sensitive isolate. Two isolates in each instance retained sensitivity against IPM, CFX and AZI (Table 7).

The enhanced antibiotic resistance might be due to several factors including the limited diffusion through the biofilm, enzyme mediated resistance, interaction and neutralization of the antimicrobial substance by the biofilm, metabolic state of the organisms in the biofilm, genetic adaptation, outer membrane structure and efflux pump. The data of the present study suggested that multispecies biofilm may actively take part in the frequent spreading of antibiotic resistance gene in the natural

### Table 3: Antibiotics susceptibility pattern of the isolates from mud

| Antibiotics | TRI-SUL | ERY | CEF | CIP | AMP | TET | CHO | CFX | POL-B | IPM | GEN | NAL | AZI |
|-------------|---------|-----|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|
| Bulk sample |         |     |     |     |     |     |     |     |       |     |     |     |     |
| *E. coli*   | R       | S   | ND  | S   | S   | R   | S   | S   | ND    | R   | S   | R   | S   |
| *Klebsiella* spp. | S   | S   | ND  | R   | S   | R   | S   | S   | ND    | S   | R   | S   | S   |
| *Salmonella* spp. | R   | S   | S   | R   | S   | R   | S   | ND  | ND    | ND  | S   | S   | ND  |
| *Pseudomonas* spp. | S   | ND  | S   | S   | S   | S   | S   | ND  | S     | S   | S   | S   |
| *Bacillus* spp. | S   | S   | S   | ND  | R   | S   | S   | ND  | S     | S   | S   | S   |
| *Staphylococcus* spp. | R   | S   | R   | R   | R   | S   | ND  | S   | ND    | S   | ND  | S   |
| Biofilm     |         |     |     |     |     |     |     |     |       |     |     |     |     |
| *E. coli*   | R       | R   | ND  | R   | R   | R   | R   | ND  | R     | R   | R   | R   | R   |
| *Klebsiella* spp. | R   | R   | ND  | R   | R   | R   | R   | ND  | R     | R   | R   | R   |
| *Salmonella* spp. | R   | R   | R   | R   | R   | R   | R   | ND  | R     | R   | R   | R   |
| *Pseudomonas* spp. | S   | ND  | R   | R   | R   | R   | R   | ND  | R     | R   | R   | R   |
| *Bacillus* spp. | R   | R   | ND  | R   | R   | R   | R   | ND  | R     | R   | R   | R   |
| *Staphylococcus* spp. | R   | R   | ND  | R   | R   | R   | R   | ND  | R     | R   | R   | R   |

**ND: Not done; R: Resistant; S: Sensitive**

TRI-SUL= Trimethoprim/sulfamethoxazole (25 µg); ERY= Erythromycin (15 µg); CEF= Ceftriaxone (30 µg); CIP= Ciprofloxacin (5 µg); AMP= Ampicillin (10 µg), TET= Tetracycline (30 µg), CHO= Chloramphenicol (30 µg), CFX= Cefixime (5 µg), POL-B= Polymyxin B (300 units), IPM= Imipenem (10 µg), GEN= Gentamicin (10 µg), NAL= Nalidixic acid (30 µg), AZI= Azithromycin (15 µg).

### Table 4: Antibiotics susceptibility pattern of the isolates from municipal waste water

| Antibiotics | TRI-SUL | ERY | CEF | CIP | AMP | TET | CHO | CFX | POL-B | IPM | GEN | NAL | AZI |
|-------------|---------|-----|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|
| Bulk sample |         |     |     |     |     |     |     |     |       |     |     |     |     |
| *E. coli*   | R       | S   | ND  | S   | S   | R   | S   | S   | ND    | R   | S   | R   | S   |
| *Klebsiella* spp. | S   | S   | ND  | R   | S   | R   | S   | S   | ND    | R   | S   | R   | S   |
| *Salmonella* spp. | R   | S   | S   | R   | S   | R   | S   | ND  | ND    | ND  | S   | S   | ND  |
| *Pseudomonas* spp. | S   | ND  | S   | S   | S   | S   | S   | ND  | S     | S   | S   | S   |
| *Bacillus* spp. | S   | S   | S   | ND  | R   | S   | S   | ND  | S     | S   | S   | S   |
| *Staphylococcus* spp. | R   | S   | R   | R   | R   | S   | ND  | S   | ND    | S   | ND  | S   |
| Biofilm     |         |     |     |     |     |     |     |     |       |     |     |     |     |
| *E. coli*   | R       | R   | ND  | R   | R   | R   | R   | ND  | R     | R   | R   | R   | R   |
| *Klebsiella* spp. | R   | R   | ND  | R   | R   | R   | R   | ND  | R     | R   | R   | R   |
| *Salmonella* spp. | R   | R   | R   | R   | R   | R   | R   | ND  | R     | R   | R   | R   |
| *Pseudomonas* spp. | S   | ND  | R   | R   | R   | R   | R   | ND  | R     | R   | R   | R   |
| *Bacillus* spp. | R   | R   | ND  | R   | R   | R   | R   | ND  | R     | R   | R   | R   |
| *Staphylococcus* spp. | R   | R   | ND  | R   | R   | R   | R   | ND  | R     | R   | R   | R   |

**ND: Not done; R: Resistant; S: Sensitive**

TRI-SUL= Trimethoprim/sulfamethoxazole (25 µg); ERY= Erythromycin (15 µg); CEF= Ceftriaxone (30 µg); CIP= Ciprofloxacin (5 µg); AMP= Ampicillin (10 µg), TET= Tetracycline (30 µg), CHO= Chloramphenicol (30 µg), CFX= Cefixime (5 µg), POL-B= Polymyxin B (300 units), IPM= Imipenem (10 µg), GEN= Gentamicin (10 µg), NAL= Nalidixic acid (30 µg), AZI= Azithromycin (15 µg).
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### Table 5: Antibiotics susceptibility pattern of the isolates from pharmaceutical waste water

| Antibiotics | Isolates | TRI-SUL | ERY | CEF | CIP | AMP | TET | CHO | CFX | POL-B | IPM | GEN | NAL | AZI |
|-------------|----------|---------|-----|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|
| Bulk sample | *Klebsiella* spp. | R | S | ND | R | R | R | S | S | ND | S | S | R | S |
|             | *Pseudomonas* spp. | S | ND | S | S | S | S | S | ND | S | S | R | S |
|             | *Bacillus* spp. | S | S | ND | S | S | S | S | ND | R | S | S | S | ND |
|             | *Staphylococcus* spp. | S | R | S | S | S | S | S | ND | S | S | ND | R |
| Biofilm     | *Klebsiella* spp. | R | R | ND | R | R | R | R | S | ND | R | S | R | S |
|             | *Pseudomonas* spp. | R | ND | R | R | R | R | S | ND | S | R | S | R | S |
|             | *Bacillus* spp. | R | R | ND | R | R | R | R | ND | R | R | S | R | ND |
|             | *Staphylococcus* spp. | R | S | R | R | R | R | ND | R | ND | S | ND | R |

ND: Not done; R: Resistant; S: Sensitive
TRI-SUL= Trimethoprim/sulfamethoxazole (25 µg); ERY= Erythromycin (15 µg); CEF= Ceftriaxone (30 µg); CIP= Ciprofloxacin (5 µg); AMP= Ampicillin (10 µg); TET= Tetracycline (30 µg); CHO= Chloramphenicol (30 µg); CFX= Cefixime (5 µg); POL-B= Polymyxin B (300 units); IPM= Imipenem (10 ½g); GEN= Gentamicin (10 µg); NAL= Nalidixic acid (30 µg); AZI= Azithromycin (15 µg).

### Table 6: Antibiotics susceptibility pattern of the isolates from hospital waste water

| Antibiotics | Isolates | TRI-SUL | ERY | CEF | CIP | AMP | TET | CHO | CFX | POL-B | IPM | GEN | NAL | AZI |
|-------------|----------|---------|-----|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|
| Bulk sample | *Klebsiella* spp. | S | S | ND | R | S | S | R | S | ND | R | S | S | S |
|             | *Pseudomonas* spp. | ND | ND | S | S | S | S | S | S | ND | S | S | R | S |
|             | *Bacillus* spp. | S | S | ND | S | R | S | S | ND | S | S | S | ND |
|             | *Staphylococcus* spp. | S | S | S | S | S | S | S | ND | R | ND | S | ND | S |
| Biofilm     | *Klebsiella* spp. | R | R | ND | R | R | R | R | S | ND | R | S | R | R |
|             | *Pseudomonas* spp. | ND | ND | R | R | R | R | R | S | ND | S | S | R | S |
|             | *Bacillus* spp. | R | R | ND | R | R | R | R | ND | R | R | S | R | ND |
|             | *Staphylococcus* spp. | R | R | R | R | R | R | ND | R | ND | S | ND | R |

ND: Not done; R: Resistant; S: Sensitive
TRI-SUL= Trimethoprim/sulfamethoxazole (25 µg); ERY= Erythromycin (15 µg); CEF= Ceftriaxone (30 µg); CIP= Ciprofloxacin (5 µg); AMP= Ampicillin (10 µg); TET= Tetracycline (30 µg); CHO= Chloramphenicol (30 µg); CFX= Cefixime (5 µg); POL-B= Polymyxin B (300 units); IPM= Imipenem (10 ½g); GEN= Gentamicin (10 µg); NAL= Nalidixic acid (30 µg); AZI= Azithromycin (15 µg).

### Table 7: Antibiotics susceptibility pattern of the isolates from domestic waste

| Antibiotics | Isolates | TRI-SUL | ERY | CEF | CIP | AMP | TET | CHO | CFX | POL-B | IPM | GEN | NAL | AZI |
|-------------|----------|---------|-----|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|
| Bulk sample | *E. coli* | S | S | ND | S | S | S | S | S | ND | R | S | R | S |
|             | *Klebsiella* spp. | S | S | ND | S | R | R | S | S | ND | S | S | S | S |
|             | *Salmonella* spp. | S | S | S | S | R | S | S | ND | ND | R | S | S | ND |
|             | *Pseudomonas* spp. | R | ND | S | S | S | R | S | S | ND | S | S | R | S |
|             | *Bacillus* spp. | R | R | v | S | S | S | S | ND | S | S | S | ND |
|             | *Staphylococcus* spp. | S | R | S | S | S | R | S | ND | R | ND | S | ND | S |
| Biofilm     | *E. coli* | R | S | ND | R | R | R | R | S | ND | S | S | R | R |
|             | *Klebsiella* spp. | R | R | ND | R | R | R | R | S | ND | R | S | R | S |
|             | *Salmonella* spp. | S | R | R | S | S | R | R | ND | R | ND | S | S | ND |
|             | *Pseudomonas* spp. | R | ND | R | R | R | R | R | S | ND | S | S | R | S |
|             | *Bacillus* spp. | R | R | ND | R | R | R | R | ND | R | S | R | ND |
|             | *Staphylococcus* spp. | R | R | R | R | R | R | ND | R | ND | S | ND | R |

ND: Not done; R: Resistant; S: Sensitive
TRI-SUL= Trimethoprim/sulfamethoxazole (25 µg); ERY= Erythromycin (15 µg); CEF= Ceftriaxone (30 µg); CIP= Ciprofloxacin (5 µg); AMP= Ampicillin (10 µg); TET= Tetracycline (30 µg); CHO= Chloramphenicol (30 µg); CFX= Cefixime (5 µg); POL-B= Polymyxin B (300 units); IPM= Imipenem (10 ½g); GEN= Gentamicin (10 µg); NAL= Nalidixic acid (30 µg); AZI= Azithromycin (15 µg).
environment which will result in extended frequency of MDR cases and hence expand the public health risk.

Conclusion
The findings of the present study revealed that the samples from surface water, mud and different wastes contained different types of pathogenic microbes including MDR bacteria. Due to the irrational use of the surface water to serve different purposes such as bathing, washing food items, household uses etc. and the unplanned disposal of industrial, hospital and domestic wastes, these pathogenic multidrug resistance bacteria may get entry into the environment, foods or water supply systems. Due to the presence of multi-species biofilms, these may result in the drastic dissemination of MDR bacteria. Such possibilities have been justified in this study, as the multi-species biofilm formed in vitro from the same samples were found to enhance the antibiotic resistance. Most of the sensitive isolates were found to develop antibiotic resistance when they were isolated from biofilms. Thus the outcome of the present study vastly draws the attention for the safe disposal of industrial, hospital and municipal wastes.

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Conflict of interest
Authors have no potential conflicts of interest.

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