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Research Article
Characterization of Harmful Microorganisms Residing Within Pharmaceutical Wastes and Detection of Their Enhanced Drug-Resistance Traits

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Abstract

Background and Objective: Waste management is becoming a vital environmental issue now a day. Present study attempted to figure out harmful microorganisms in both treated and untreated pharmaceutical wastes, the drug-resistance pattern of the microbial isolates from the selected wastes, the anti-bacterial activity of the waste samples and the increased bacterial resistance against different antimicrobial agents possibly triggered by the biofilm formation. **Materials and Methods:** Isolation and identification of pathogenic microorganisms was accomplished through conventional and cultural methods and the disc diffusion (Kirby-Bauer test) method was intruded for antibiogram and antimicrobial activity measurement. **Results:** The total viable bacteria and fungi was estimated up to 10^6 and 10^3 CFU mL^{-1}, respectively in the untreated wastes while the microbial load elevated up to 10^8 and 10^6 CFU mL^{-1}, respectively in the treated waste samples. While the bacterial load was noticed to be increased in all the treated wastes up to a quantity of 10^5 CFU mL^{-1}, only four antibiotics out of twelve were found to be effective against the isolates. Interestingly, after forming biofilms, all the isolates showed resistance against the drugs tested. The untreated wastes unveiled their massive anti-bacterial traits against the soil bacteria isolated from the surroundings of pharmaceuticals. **Conclusion:** The research projected on the continuous exposure of the environment to a range of antibiotics and the drug-resistance genes within the environmentally available microbial consortia posing dreadful impact on public health.

Key words: Pharmaceutical wastes, drug resistance, multi-drug resistance (MDR), pathogenic microorganisms, biofilm, public health

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Industrial activities are saturated with the concomitant production of wastes and the amount of toxicity of the excreted wastes varies with the types of industrial processes. Pharmaceutical and tannery effluents (both liquid and solid) are ranked as the highest pollutants within the environment[1,2]. In developing countries like Bangladesh, both the large (drug manufacturing companies) or small industries (for example, research laboratories) generate a considerable amount of solid and liquid wastes especially through the discharge of the untreated effluents directly into the environment. The released wastes are likely to harbor pollutants and heavy metals like cadmium, chromium, lead, nickel, zinc and copper, residual antibiotics, analgesics, antiseptics, lipid-lowering drugs, synthetic estrogens and anti-inflammatory drugs, etc. These toxic chemicals ultimately cause toxicity towards the plants and wildlife, fish and obviously soils and waters[2]. Dissemination of pathogenic and the drug-resistant microorganisms into the surrounding environment are triggered by the improper disposal of pharmaceutical wastewater[3,4]. For example, *Escherichia coli*, *Enterococcus* spp., *Pseudomonas* spp. confer resistance to sulfonamide, trimethoprim and quinolone. As these "disposed off" materials find its way into the aquatic streams or drinking water distribution systems so that these can possibly possess negative impact not only on humans and wild lives but also within the aquatic lives[4,5]. A number of studies have already explained that the exploitation of the aquatic environment is mainly caused by the infusion of pharmaceuticals and agricultural wastes into the nearby aquatic source[5-7].

It's already evident from the previous studies that the Multi Drug Resistant (MDR) bacteria propagated through pharmaceutical solid wastes are capable in transmitting the drug-resistance gene(s) into the antibiotic sensitive strains[4,8]. It's also interesting to note that the pharmaceutical wastewaters may help to form biofilm of the drug-resistant bacteria through the eventual activation of the drug-resistance genes within the bacterial consortium. Thus, the aquatic ecosystems receiving waste waters can act as reservoir of the drug-resistance genes, which could potentially be transferred to the susceptible bacterial strains. This may result in the transformation of the non-pathogenic strains into the pathogenic ones[1,4]. Moreover, the accumulation of active pharmaceutical ingredients (used for the manufacturing of antibiotics) may affect the selection of bacterial consortium to be embedded into the biofilm as well as to confer the drug-resistance trait. Thus, the wastewater treatment plants or the effluent treatment plants may serve as the ideal place for horizontal transfer of the drug-resistance gene(s) due to the intense amount of such microorganisms[4]. Hence, the pharmaceutical wastes and the microbial biofilm have become linked with the failure to control or eliminate them by antibiotic and the biocide regimes or by the chemical effluent treatment[9,10]. Apparently, the biofilm sample collected from the untreated effluents from the pharmaceuticals may develop 10-1,000 times resistance against specific antibiotic compared with their planktonic counterparts[10-12]. Recent studies have demonstrated that biofilms of aquatic and soil ecosystems adjacent to the pharmaceutical industries harbor the drug-resistant bacteria which may develop the Quorum-Sensing (QS) regulated mechanisms as well as the conventional resistance mechanisms like the β-lactamase exposure or the upregulated efflux pumps to exhale the antibiotic from bacterial cytosol[5,13]. It's not unlikely that the Multi Drug Resistant (MDR) or the Extensively Drug Resistant (XDR) bacteria evolved from such environmental dysbiosis can raise the public health fatality; thereby demanding the significance of the proper management of pharmaceutical wastes[4,9]. Thus it's important for the pharmaceutical companies to strictly adhere to exact guidelines to conduct the waste management properly along with the implementation of Good Manufacturing Practice (GMP) as well as the Total Quality Management (TQM)[14]. A nearly similar study previously conducted was successfully able to establish that the disposal of industrial waste into the environment has huge negative impact on the ecological balance such as supporting the development of the resistant bacteria as well as the resistant gene transfer consequence from resistant strain to sensitive strain through conjugation[4]. Present study further chalked out a complete microbiological profile of the pharmaceuticals wastes, the drug resistance pattern of the bacterial isolates and the demonstration of the *in vitro* antimicrobial activity of the pharmaceutical wastes.

MATERIALS AND METHODS

Study area, sampling and sample processing: A total of 20 liquid effluent samples were analyzed: 10 samples were collected before treated by effluent treatment plant and the other 10 samples were collected after treating with the effluent treatment plant. Samples were collected randomly from several renowned pharmaceuticals in Bangladesh during October, 2016 to December, 2017 following the standard protocol[4,15]. For the identification and enumeration of pathogenic bacteria and fungi, 1 mL of each sample was diluted up to 10^{-4} according to the standard guideline[16].

Microbiological analysis pharmaceutical waste: For each of the cases, 0.1 mL of sample from the dilution 10^{-2} and 10^{-4} was introduced on to the Luria Bertani (LB) agar and Sabouraud Dextrose Agar (SDA) for the isolation of total viable bacteria and fungi. Additionally, to enumerate some specific bacteria like *Staphylococcus* spp., *Pseudomonas* spp., *Vibrio* spp., *Bacillus* spp. and Actinomycetes, several selective media was prepared such as Mannitol Salt agar, Cetrimide agar, TCBS agar, Starch agar and Actinomycetes agar consecutively. MacConkey agar was used to confirm the presence of coliforms (*E. coli* and *Klebsiella* spp.). Finally, the standard biochemical tests were performed for the confirmative identification of all the pathogenic isolates found in the samples[4].

Antibiotic susceptibility test of the isolates: The standard agar-disc-diffusion method (Kirby Bauer technique) was used to examine the antibiotic susceptibility of the isolates (either sensitive or resistant) on Mueller-Hinton Agar (MHA) (Difco, Detroit, MI)[4,17]. In this study, 12 commonly available antibiotics were used such as Ampicillin (AMP 10 µg), Tetracycline (TER 30 µg), Azithromycin (AZI 15 µg), Penicillin (PEN 10 µg), Gentamicin (GEN 10 µg), Streptomycin (STP 10 µg), Erythromycin (15 µg), Ciprofloxacin (CIP 5 µg), Ceftriaxone (CEF 30 µg), Cefixime (CFX 5 µg), Imipenem (IPM 30 µg) and Chloramphenicol (CHL 10 µg). Among 12 antibiotics, only Penicillin (PEN 10 µg) was narrow spectrum. However, rest of the antibiotics was broad spectrum. All plates were then incubated at 37°C for 24 hrs. After incubation, the plates were examined and the zone of inhibition was measured in mm[4].

Isolation of soil bacteria from pharmaceutical surroundings: Around 10 g of soil samples were collected from the selected areas of the pharmaceuticals industries and were homogenized with 90 mL of distilled water. Samples were then diluted up to 10^{-8} and 0.1 mL of each samples from the dilutions 10^{-6} and 10^{-8} were spread on to different selective media like Mannitol Salt agar (MSA), Cetrimide (CM) agar, Thio-citrate Bile Salts Sucrose (TCBS) agar, starch agar and Actinomycetes agar to observe the growth of *Staphylococcus* spp., *Pseudomonas* spp., *Vibrio* spp. *Bacillus* spp. and actinomycetes, consecutively[16].

Antimicrobial assay: For the determination of anti-microbial activity, modified agar well diffusion method was employed using MHA plates[4]. Suspensions of different bacterial strains (isolated from the soil samples of the nearby the pharmaceuticals areas); i.e., *E. coli*, *Pseudomonas* spp., *Vibrio* spp., *Staphylococcus aureus*, *Bacillus* spp. and *Salmonella* spp. were introduced on to the MHA were prepared using normal saline, consisting of 10^6 CFU mL^{-1} with a turbidity equivalent to that of the 0.5 mL McFarland standard and each suspension was then subject to lawn on the MHA. Wells were dug (8 mm^3) on the inoculated MHA media which allow approximately 100 µL or 11 mg mL^{-1} of samples to fill up. Normal saline was used as negative controls whereas antibiotic disk of Gentamicin (GEN 10 µg) was used as positive control. Plates were incubated at 37°C overnight and examined for the zone of inhibition. The diameter of the inhibition zone was measured in mm using slide calipers.

Development of resistant genes in natural biofilm

Collection and processing of biofilm: For launching the *in vitro* resistant pattern of biofilm-originated bacteria, samples were collected from different wastewater reservoir tanks discharged from the pharmaceutical industries. Subsequently, the samples were transferred aseptically in sterile screw capped bottles within 1 hr of collection into the laboratory and immediately subjected to microbiological analysis. Same bacterial strains were isolated from biofilm through conventional culture techniques as described above. Afterward, all the isolates were further confirmed biochemically[4].

Detection of drug resistant pattern of biofilm associated isolates: According to the disc-diffusion method, one loop full culture of each bacterial isolates were further suspended into the LB broth and after incubation the bacterial inoculum was prepared to lawn on to MHA to examine the potency of the same antibiotics to inhibit the growth of biofilm associated isolates[4]. Plates were then inverted and incubated at 37°C for 24 hrs. After incubation, the zone of inhibition was measured and the susceptibility pattern was detected as prescribed earlier[18].

RESULTS

Frequency of microorganisms in pharmaceutical waste samples studied: In case of the untreated samples the microbial cell number of each samples were noticed to be comparatively lower than the treated ones. However, the existence of some undesirable microflora was very prominent in maximum untreated samples over treated waste samples (Table 1). The contamination rate of total viable bacteria and

Table 1: Microbiological profile of pharmaceutical wastes

Category of sample	Number of samples	State	TVB	Fungi	*Klebsiella* spp.	*E. coli*	*Vibrio* spp.	*Pseudomonas* spp.	*Bacillus* spp.	*Staphylococcus* spp.	*Shigella* spp.
Before treatment	1	Liquid	1.1×10^{6}	1.7×10^{3}	0	2.1×10^{3}	3.1×10^{2}	1.9×10^{3}	4.9×10^{3}	1.1×10^{2}	3.3×10^{3}
	2	Liquid	4.5×10^{5}	6.0×10^{2}	$6.\times10^{2}$	6.7×10^{3}	3.7×10^{3}	3.7×10^{3}	4.7×10^{2}	8.0×10^{4}	2.7×10^{2}
	3	Liquid	8.7×10^{6}	1.4×10^{3}	0	1.9×10^{3}	3.9×10^{3}	3.9×10^{3}	2.9×10^{3}	4.3×10^{3}	3.9×10^{3}
	4	Liquid	1.1×10^{5}	0	0	2.9×10^{2}	0	2.1×10^{2}	5.1×10^{3}	3.7×10^{2}	2.0×10^{3}
	5	Liquid	7.1×10^{6}	5.1×10^{3}	0	5.0×10^{3}	0	3.0×10^{3}	3.9×10^{3}	5.9×10^{3}	0
	6	Liquid	1.5×10^{6}	1.1×10^{3}	0	1.8×10^{3}	0	2.8×10^{3}	3.8×10^{3}	2.1×10^{3}	0
	7	Liquid	3.5×10^{6}	6.0×10^{2}	3.7×10^{3}	4.0×10^{2}	0	2.0×10^{2}	3.0×10^{3}	3.0×10^{3}	0
	8	Liquid	8.0×10^{6}	6.0×10^{3}	5.5×10^{3}	3.0×10^{3}	3.8×10^{3}	3.2×10^{3}	5.2×10^{3}	3.7×10^{3}	4.3×10^{3}
	9	Liquid	1.0×10^{5}	2.2×10^{3}	4.8×10^{3}	2.9×10^{3}	2.7×10^{3}	1.8×10^{3}	3.8×10^{3}	3.0×10^{3}	3.7×10^{2}
	10	Liquid	2.1×10^{6}	1.4×10^{3}	2.9×10^{3}	1.5×10^{3}	3.5×10^{3}	1.0×10^{3}	1.9×10^{3}	5.0×10^{3}	5.9×10^{3}
After treatment	1	Liquid	5.9×10^{8}	1.1×10^{5}	0	0	2.0×10^{5}	0	0	0	0
	2	Liquid	2.6×10^{8}	4.0×10^{5}	0	0	0	0	0	3.6×10^{4}	0
	3	Liquid	2.1×10^{8}	0	5.7×10^{5}	0	0	0	5.6×10^{4}	1.1×10^{5}	0
	4	Liquid	5.6×10^{8}	5.6×10^{3}	6.5×10^{5}	2.0×10^{5}	0	1.1×10^{4}	0	0	2.0×10^{5}
	5	Liquid	1.1×10^{7}	1.1×10^{5}	2.8×10^{5}	1.9×10^{4}	0	4.0×10^{5}	4.5×10^{5}	2.3×10^{5}	3.0×10^{5}
	6	Liquid	6.9×10^{8}	2.9×10^{5}	1.9×10^{5}	0	0	6.0×10^{4}	0	5.0×10^{4}	0
	7	Liquid	2.9×10^{8}	3.6×10^{5}	3.0×10^{4}	4.6×10^{5}	0	6.7×10^{5}	0	6.9×10^{4}	0
	8	Liquid	2.9×10^{8}	4.1×10^{5}	0	5.1×10^{5}	3.9×10^{5}	2.0×10^{5}	2.9×10^{4}	2.1×10^{5}	0
	9	Liquid	4.6×10^{8}	5.0×10^{6}	0	0	1.9×10^{5}	0	1.9×10^{5}	3.4×10^{4}	0
	10	Liquid	6.1×10^{7}	5.7×10^{4}	0	2.7×10^{4}	3.0×10^{4}	0	6.7×10^{5}	7.0×10^{4}	0

All the experiments have been done three times and the results were reproducible. One representative data have been shown. Actinomycetes were totally absent in each category of samples. TVB: Total viable bacteria

fungi in untreated sample was noticed within the range of 1×10^5-8.7×10^6 and 6×10^2-6×10^3 CFU mL^{-1}, respectively, whereas in treated samples the rate was estimated within the range of 10^7-10^8 and 10^3-10^6 CFU mL^{-1}, respectively. All the untreated samples were found to be contaminated with *E. coli*, *Pseudomonas* spp., *Bacillus* spp. and *Staphylococcus* spp. within the range of 10^2-10^3 CFU mL^{-1} whereas in case of treated *E. coli* was found in samples 4, 5 ,7, 8 and sample 10 (up to 10^5 CFU mL^{-1}); *Pseudomonas* spp. was found in samples 4-7 and in sample 10 (up to 10^5 CFU mL^{-1}); *Bacillus* spp. was found in samples 3, 5, 8-10 (up to 10^5 CFU mL^{-1}) and *Staphylococcus* spp. was found in samples 2, 3, 5-10 (up to 10^5 CFU mL^{-1}) given in Table 1.

In case of the treated samples the proliferation of *Klebsiella* spp. was noticed in samples 2, 7-10 (up to 10^3 CFU mL^{-1}); *Vibrio* spp. was enumerated in samples 1, 2, 3, 8-10 (up to 10^3 CFU mL^{-1}) and the existence of *Shigella* spp. was demonstrated in samples 1, 2, 3, 4, 8-10 (up to 10^3 CFU mL^{-1}). Subsequently, in the treated waste samples, *Klebsiella* spp. was found to dominate in samples 2, 7-10 (up to 10^5 CFU mL^{-1}), *Vibrio* spp. in samples 1 and in samples 8-10 (up to 10^5 CFU mL^{-1}) and *Shigella* spp. in samples 4 and 5 up to 10^5 CFU mL^{-1} (Table 1).

Antibiogram of different pathogenic isolates: All the isolates from both categories (treated and untreated, respectively) showed their susceptibility patterns: some were resistant and some were sensitive given in Table 2. In case of untreated sample *Klebsiella* spp. showed 100% resistance against AMP, CIP, STE, PEN, TER, CHL and the 100% sensitivity to CEF, IPM, GEN, AZI, CFX, ERY. *Bacillus* spp. showed 100% resistance against AMP, CIP, STE, CEF, PEN and 100% sensitivity towards IPM, GEN, AZI, TER, CFX, ERY, CHL. *Vibrio* spp. exhibited 100% resistance against AMP, STE, PEN, TER and were 100% sensitive towards CIP, CEF, IPM, GEN, AZI, CFX, ERY, CHL. *Staphylococcus* spp. exhibited 100% resistance against AMP, CIP, STE, PEN, TER and 100% sensitivity towards AMP, CEF, IPM, GEN, AZI, CFX, ERY, CHL. *E. coli* was found to be 100% resistant against AMP, CIP, STE, CEF, PEN while 100% sensitive to IPM, GEN, AZI, TER, CFX, ERY, CHL. *Pseudomonas* spp. showed 100% resistance against AMP, CIP, STE, CEF, IPM, PEN, GEN, AZI, TER and 100% sensitivity towards ERY, CHL. *Shigella* spp. was found to be resistant against AMP, CIP, STE, PEN, TER and 100% sensitive to CEF, IPM, GEN, AZI, CFX, ERY, CHL (Table 2).

In case of the treated samples, *Klebsiella* spp. showed 100% resistance against AMP, PEN, TER, CHL and 100% sensitivity towards CIP, STE, CEF, IPM, GEN, AZI, CFX, ERY, CHL. *Bacillus* spp. showed 100% resistance against AMP, STE, PEN and 100% sensitivity towards CIP, CEF, IPM, GEN, AZI, TER, CFX, ERY, CHL. *Vibrio* spp. exhibited 100% resistance against AMP, STE, PEN, TER and 100% sensitivity towards CIP, CEF, IPM, GEN, AZI, CFX, ERY, CHL. *Staphylococcus* spp. exhibited 100% resistance against AMP, STE, PEN, TER while they were 100% sensitive to AMP, CIP, CEF, IPM, GEN, AZI, CFX, ERY, CHL. *E. coli* was found to be 100% resistant against AMP, STE, PEN and 100% sensitive against CIP, CEF, IPM, GEN, AZI, TER, CFX, ERY, CHL. *Pseudomonas* spp. showed 100% resistance against AMP, CIP, STE, PEN, TER and 100% sensitive towards CIP, CEF, IPM, GEN, AZI, CFX, ERY, CHL. *Shigella* spp. was found to be resistant against AMP, STE, PEN, TER while they were 100% sensitive against CIP, CEF, IPM, GEN, AZI, CFX, ERY, CHL (Table 2).

Enumeration of soil bacteria from the pharmaceutical surroundings: Current study attempted to analyze the bacterial profile of the pharmaceutical surroundings. *E. coli*, *Salmonella* spp., *Pseudomonas* spp., *Bacillus* spp., *Vibrio* spp. and *Staphylococcus* spp. were noticed along the 20 different sites of agricultural territory nearby the pharmaceutical industries in Table 3.

Anti-bacterial activity of pharmaceutical waste samples: Each of the samples of both categories (treated sample and untreated pharmaceutical waste sample) showed antimicrobial activity against almost all organisms given in Table 4. In case of the untreated samples the anti-bacterial activity was found to be very high against the tested bacteria rather than the treated samples. Except sample 9, all the untreated samples showed anti-bacterial activity against *Bacillus* spp. and *Pseudomonas* spp. Samples 4, 6, 7 and 10 showed their efficacy against *E. coli*. Samples 5, 7 and 10 exhibited anti-bacterial activity against *Vibrio* spp. Growth of *Salmonella* spp. was noticed to be inhibited by samples 3, 6 and 8 whereas samples 1-4 and 7-9 showed their effectiveness against *Staphylococcus* spp. The treated samples were found to be highly effective only against *Bacillus* spp. and *Pseudomonas* spp. Samples 1-4 and 6-7 effectively eradicated the growth of *Bacillus* spp. and *Pseudomonas* spp. Sample 1 showed the highest zone 30 mm against *Staphylococcus* spp. among all the untreated samples whereas sample 1 showed the lowest zone (5 mm) against *Pseudomonas* spp.

Table 2: Antimicrobial susceptibility pattern of different pathogenic isolates found in pharmaceutical wastes

Isolates antibiotics (µg)	*Klebsiella* spp. (n = 5)		*Bacillus* spp. (n = 10)		*Vibrio* spp. (n = 6)		*Staphylococcus* spp. (n = 10)		*E. coli* (n = 10)		*Pseudomonas* spp. (n = 10)		*Shigella* spp. (n = 7)	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Before treatment (%)														
AMP (10)	100	0	100	0	100	0	100	100	100	0	100	0	100	0
CIP (5)	100	0	100	0	0	100	100	0	100	0	100	0	100	0
STE(10)	100	0	100	0	100	0	100	0	100	0	100	0	100	0
CEF (30)	0	100	100	0	0	100	0	100	100	0	100	0	0	100
IPM (30)	0	100	0	100	0	100	0	100	0	100	100	0	0	100
PEN (10)	100	0	100	0	100	0	100	0	100	0	100	0	100	0
GEN (10)	0	100	0	100	0	100	0	100	0	100	100	0	0	100
AZI (15)	0	100	0	100	0	100	0	100	0	100	100	0	0	100
TER (30)	100	0	0	100	100	0	100	0	0	100	100	0	100	0
CFX (5)	0	100	0	100	0	100	0	100	0	100	0	0	0	100
ERY (15)	0	100	0	100	0	100	0	100	0	100	0	100	0	100
CHL (10)	100	0	0	100	0	100	0	100	0	100	0	100	0	100
Isolates antibiotics (µg)	*Klebsiella* spp. (n = 5)		*Bacillus* spp. (n = 10)		*Vibrio* spp. (n = 4)		*Staphylococcus* spp. (n = 8)		*E. coli* (n = 5)		*Pseudomonas* spp. (n = 5)		*Shigella* spp. (n = 2)	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S
After treatment (%)														
AMP (10)	100	0	100	0	100	0	100	100	100	0	100	0	100	0
CIP (5)	0	100	0	100	0	100	0	100	0	100	100	100	0	100
STE (10)	0	100	100	0	100	0	100	0	100	0	100	0	100	0
CEF (30)	0	100	0	100	0	100	0	100	0	100	0	100	0	100
IPM (30)	0	100	0	100	0	100	0	100	0	100	0	100	0	100
PEN (10)	100	0	100	0	100	0	100	0	100	0	100	0	100	0
GEN (10)	0	100	0	100	0	100	0	100	0	100	0	100	0	100
AZI (15)	0	100	0	100	0	100	0	100	0	100	0	100	0	100
TER (30)	100	0	0	100	100	0	100	0	0	100	100	0	100	0
CFX (5)	0	100	0	100	0	100	0	100	0	100	0	100	0	100
ERY (15)	0	100	0	100	0	100	0	100	0	100	0	100	0	100
CHL (10)	100	0	0	100	0	100	0	100	0	100	0	100	0	100

AMP: Ampicillin, CIP: Ciprofloxacin, CEF: Ceftriaxone, IPM: Imipenem, ERY: Erythromycin, AZI: Azithromycin, GEN: Gentamicin, PEN: Penicillin, TER: Tetracycline, CFX: Cefixime, STE: Streptomycin, CHL: Chloramphenicol, N: Number of isolates, R: Resistant, S: Sensitive

Table 3: Detection of important soil bacteria from the pharmaceutical surroundings

Sample	*E. coli*	*Salmonella* spp.	*Pseudomonas* spp.	*Bacillus* spp.	*Vibrio* spp.	*Staphylococcus* spp.
1	+	+	+	+	-	+
2	+	+	+	+	-	+
3	+	-	+	+	-	+
4	-	-	+	+	-	+
5	-	-	+	+	-	+
6	-	-	+	+	-	+
7	+	-	+	+	+	+
8	+	-	+	+	+	+
9	-	+	+	+	+	+
10	+	+	+	+	+	+
11	+	+	+	+	-	+
12	+	+	+	+	-	+
13	+	-	+	+	-	+
14	-	-	+	+	-	+
15	-	-	+	+	-	+
16	-	-	+	+	-	+
17	+	-	+	+	+	+
18	+	-	+	+	+	+
19	-	+	+	+	+	+
20	+	+	+	+	+	+

Table 4: Antimicrobial activity of the pharmaceutical effluent against the beneficial soil bacteria isolated from the pharmaceutical surroundings

Samples categories	Number of samples	State	*E. coli*	*Vibrio* spp.	*Bacillus* spp.	*Pseudomonas* spp.	*Salmonella* spp.	*Staphylococcus* spp.
Before treatment	1	Liquid	-	-	12 mm	20 mm	-	30 mm
	2	Liquid	-	-	8 mm	10 mm	-	8 mm
	3	Liquid	-	-	12 mm	10 mm	10 mm	20 mm
	4	Liquid	20 mm	-	10 mm	12 mm	-	10 mm
	5	Liquid	-	10 mm	10 mm	14 mm	-	-
	6	Liquid	12 mm	-	12 mm	15 mm	20 mm	-
	7	Liquid	15 mm	12 mm	8 mm	15 mm	-	10 mm
	8	Liquid	-	-	8 mm	20 mm	15 mm	15 mm
	9	Liquid	-	-	-	-	-	12 mm
	10	Liquid	15 mm	13 mm	17 mm	14 mm	-	-
After treatment	1	Liquid	-	-	6 mm	5 mm	-	-
	2	Liquid	-	-	9 mm	6 mm	-	-
	3	Liquid	-	-	7 mm	8 mm	-	-
	4	Liquid	-	-	6 mm	8 mm	-	-
	5	Liquid	-	-	-	-	-	-
	6	Liquid	-	-	10 mm	5 mm	-	-
	7	Liquid	-	-	7 mm	-	-	-
	8	Liquid	-	-	-	-	-	-
	9	Liquid	-	-	-	-	-	-
	10	Liquid	-	-	-	-	-	-

Role of biofilm oriented microbial community to enhance the bacterial resistance: All the isolates from biofilm of waste discharge tank were found to be 100% resistant against same antibiotics tested such as AMP, CIP, CEF, IPM, ERY, AZI, GEN, PEN, TER, CFX, STE, CHL. However, before biofilm-formation, GEN, CIP, IPM and AZI were found to be most effective drugs against the same bacterial species, which evidently indicated the active influence of biofilm for the development of drug-resistance given in Table 5.

Detection of Multi Drug Resistant (MDR) bacteria from biofilm: Among all the isolated bacteria, *Pseudomonas* spp., *Bacillus* spp., *E. coli*, *Staphylococcus* spp., *Klebsiella* spp. and *Vibrio* spp. showed their resistance against multiple drug combination such as AMP, GEN given in Table 6. *Pseudomonas* spp., *Bacillus* spp. and *Staphylococcus* spp. were found to be resistant against the drug combination CIP, CEF, AZI and CHL. The combination of IPM, ERY, AZI, CIP, GEN and STE were also ineffective against *Pseudomonas* spp., *Bacillus* spp. and *Staphylococcus* spp. (Table 6). As they showed the MDR trait, they were considered pathogenic.

Table 5: Resistant phenomenon of bio-film linked bacterial strain

After bio-film formation

	Antibiotics (µg)											
Bacterial isolates	AMP (10)	CIP (10)	STE (10)	CEF (10)	IPM (10)	PEN (10)	GEN (10)	AZI (10)	TER (10)	CFX (10)	ERY (10)	CHL (10)
Klebsiella spp. (n = 5)	R	R	R	R	R	R	R	R	R	R	R	R
Bacillus spp. (n = 10)	R	R	R	R	R	R	R	R	R	R	R	R
Vibrio spp. (n = 6)	R	R	R	R	R	R	R	R	R	R	R	R
Staphylococcus spp. (n = 10)	R	R	R	R	R	R	R	R	R	R	R	R
E. coli (n = 10)	R	R	R	R	R	R	R	R	R	R	R	R
Pseudomonas spp. (n = 10)	R	R	R	R	R	R	R	R	R	R	R	R
Shigella spp. (n = 7)	R	R	R	R	R	R	R	R	R	R	R	R

The experiments have been done three times and the results were reproducible. One representative data have been shown. R: Resistant

Table 6: Detection of multi drug resistant (MDR)

Multiple drug combination	Isolates showing multi-drug resistance
AMP+GEN	*Pseudomonas* spp., *Bacillus* spp., *E. coli*, *Staphylococcus* spp., *Klebsiella* spp., *Vibrio* spp.
CIP+AZI+CHL+CFE	*Bacillus* spp., *Staphylococcus* spp., *Pseudomonas* spp.
STE+IPM+ERY+AZI+CIP+GEN+CIP	*Pseudomonas* spp, *Bacillus* spp., *Staphylococcus* spp,

Experiments have been done three times and the results were reproducible. One representative data have been shown. AMP: Ampicillin, CIP: Ciprofloxacin, CEF: Ceftriaxone, IPM: Imipenem, ERY: Erythromycin, AZI: Azithromycin, GEN: Gentamicin, PEN: Penicillin, TER: Tetracycline, CFX: Cefixime, STE: Streptomycin, CHL: Chloramphenicol

DISCUSSION

Pharmaceutical industries are supposed to treat the pharmaceutical wastes properly before discharging them into the environment; but unfortunately, especially in our country, most of the times some of these industries discharge untreated solid wastes (such as contaminated bottles, packaging materials, etc.) or contaminated liquid wastes in the environment directly. These untreated effluents may contain pathogenic microflora containing drug-resistance genes or even they may be the MDR ones[4,19]. They may interact with natural soil microflora or waterborne microflora and make the natural beneficial microflora resistant by transferring the resistance genes along with a subsequent transformation into biofilm. Recent advances explain that chronic exposure to antibiotics, even at very low concentrations can promote and maintain a pool of resistance genes in microbial communities or biofilm through horizontal transfer processes between individual cells or species[19,20-22]. Thus, the environmental flora becomes resistant against these antibiotics by forming biofilm. The resistant pathogens enter the animal livestock through soil, contaminated river water and the consumption of this animal meat affects human health[22,23]. Thus, in order to ensure health safety and to avoid the environmental pollution, the first and foremost responsibility is to maintain the management of clinical and pharmaceutical waste disposal[24]. Tons of untreated improperly treated or contaminated effluents or pharmaceutical wastes of industries are behind the reason of dissemination of pathogenic bacteria which has vast capabilities to develop biofilm in nature[9]. According to the previous research, in the developing countries like Bangladesh, these effluents may contain the endocrine-disrupting materials (such as Diclofenac, 17α-Ethinylestradiol, etc.) which directly infuse and run into nearby lagoon, rivers and streams[24,25]. As a result, this pollution causes adverse effects on wildlife, such as feminizing male fish, preventing reproduction, or triggering population collapse[4]. Moreover, some hospital wastes or wastes of research laboratories may contain heavy metals like cadmium, chromium, mercury, nickel, zinc, etc. whose presence may trigger the onset of an array of diseases[5,26].

With some few studies, previous research was successfully able to establish that the disposal of industrial waste into the environment has huge negative impact on the ecological balance such as supporting the development of the resistant bacteria as well as the resistant gene transfer consequence from resistant strain to sensitive strain through conjugation[4,27]. However, there was no substantiation in early studies regarding the huge roles of biofilm associated microbial community to accelerate the degree of bacterial resistance. Therefore, the current investigation focused on the necessity of appropriate waste treatment as well as the role of biofilm to increase the bacterial resistance in environment. Indeed, in the developing countries, the industrial discard management is still unregulated for lacking of appropriate governing bodies. Reportedly, pharmaceutical and medical wastes constitute about 5.7% of total waste and collected by Dhaka City Corporation every day. Thus the expansion of these wastes into the environment may lead to damage and even the onset of fatality among the associated community.

Moreover, untreated discharges from fertilizer industry causing metabolic impairment and fatality in the aquatic living organisms as it consists particular toxic ingredients such as metals, nitrates and ammonia[28]. Even the presence of the active pharmaceutical ingredients in waste waters from pharmaceutical factories was also observed in other Asian developing countries such as Taiwan (among 97 pharmaceuticals 41 compounds were detected) and Korea[19,21-23]. In addition, industrial effluents is one of the topmost alarm of ground water contamination and around 80% of all diseases in our neighboring country, India has been found to be straight away related to poor drinking water quality and unhygienic conditions[29]. In these aspects, this is very likely to state that the correct regulation of waste management is fully absent due to limited budget for the waste disposal together with the relevant expertise in the poor developing countries. Recent study proved that most of the industrial discharges carried toxic substances as well as contains different types of pathogenic microbes including the MDR bacteria[4]. These pathogenic multi-drug resistant bacteria may enter into the environment or receiving water bodies through the unplanned as well as inappropriate disposal of industrial effluents may cause the development of MDR bacteria by lateral gene transfer from the donor strain to recipient strain(s)[29-31].

CONCLUSION

Current study explained that the continuous exposure to antibiotics, even at very low concentrations, can promote and maintain a pool of resistance genes in the microbial communities or in the biofilms within the pharmaceutical wastes. Such disclosure on the incessant experience of the environment especially in the close physical proximity of the pharmaceutical industries towards the broad group of antibiotics as well the MDR or sometimes the XDR microorganisms would be of great health significance as well as to create the innovative ideas to maintain a sustainable environment.

SIGNIFICANCE STATEMENT

This study discovered the evolution of drug resistant genes upon antibiotic exposure from the pharmaceutical wastes which in turn can be beneficial for the innovation of the pharmaceutical waste discharge strategy which would be environmentally sustainable. Moreover, this study will help the researchers to uncover the critical areas of the biofilm forming microbial activities as well as the transformation of the drug-sensitive organisms to the drug resistant ones derived from their ecological niche of pharmaceutical wastes that many researchers were apparently not able to explore. Thus a new theory on the transmission of drug resistance trait may be arrived at the point of the waste management in the light of generating the Multi-drug Resistant (MDR) or even the Extensively Drug-resistant (XDR) microorganisms from the present work.

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