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Author(s): Saba Naeem[1], Iram Asim[1], Kashaf Junaid[2], Humaira Yasmeen[1]

Affiliation: [1]Department of Microbiology and Molecular Genetics, The Women University Multan, Pakistan.
[2]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Jouf University, Saudi Arabia

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Research Article

Antibiotic Resistance Profiling of Bacteria Isolated from Hospital Wastewater in Multan

Saba Naeem[1], Iram Asim[1], Kashaf Junaid[2] , Humaira Yasmeen[1]
1. Department of Microbiology and Molecular Genetics, The Women University Multan, Pakistan.
2. Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Jouf University, Saudi Arabia
*Corresponding author's email**:** humaira.6127@wum.edu.pk

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Abstract

Resistance against antibiotics mainly due to their misuse and overuse is an emerging health issue, worldwide. Antibiotics release active antibiotic residues in the environment during their production. Bacteria encounter these active antibiotic residues and the genes present in them; resultantly, they acquire resistance against antibiotics. The current study was conducted to determine the prevalence of multidrug-resistant (MDR) bacterial strains, isolated from hospital wastewater. Using standard procedures, bacterial resistance patterns against different classes of antibiotics were analysed and their species level identification was made. The disc diffusion method was used to determine the bacterial activity against antimicrobial agents. Clear zones were measured separately in millimeters around each disc. Five wastewater samples were collected from different drainage regions of hospitals situated in Multan. A total of 45 bacterial strains were isolated. Out of these 45 bacterial strains, 13 (29%) were found resistant against two or more than two classes of antibiotics. All the bacterial strains (100%) isolated from samples 2 and 3 were MDR. Twenty-five bacterial strains (55.5%) belonged to the *Bacillus* species and others belonged to *Enterococcus* species, *Micrococcus* species, *Staphylococcus* species, and *Streptococcus* species, respectively. The presence of resistant bacterial strains in hospital waste demands the availability of effectual treatment plants to treat the waste before it is disposed of into hospital waste lines.

1. Introduction

Antibiotics are chemically synthesized antimicrobial compounds used to treat bacterial infections. Bacterial resistance against antibiotics develops mainly due to their misuse and overuse [1]. In the treatment of animals and human beings, the main problem regarding the use of antibiotics is the development of resistant strains, which present a higher risk to both

animal and human health [2]. The inappropriate and widespread surveillance of antibiotics in human beings and animals is generally the first pathway for the wideness of bacterial strains resistant to antibiotics and a major cause for nosocomial infections. Antibiotics are excreted mainly into wastewater. These antibiotics, in combination with a high microbial biomass, make wastewater a potential habitat for the transfer of genes via horizontal gene transfer. The natural presence of resistant bacteria, along with the presence of pathogenic and non-pathogenic bacteria, forms a web of resistance that includes human beings, specifically in the hospital environment [3, 4].

Bacterial resistant genes are present on transposons and plasmids that disperse through transduction, conjugation, or transformation. The presence of resistant bacterial strains has been widely recorded in effusion, dump water, and drainage [5]. Cure houses/hospitals also harbor antibiotic resistant bacteria [4]. The amount of antibiotic resistant determinants present in hospital wastewater is higher as compared to community wastewater [6]. In hospitals, wastewater is produced via water utilization by patients and during the examination of patient blood samples, urine, and feces. Wastewater from hospital laboratories contains consortia of pathogens. The indirect or direct impact of the wastewater components can change the genetic makeup of the microbes present in it, which ultimately leads to a higher degree of antibiotic resistance in the bacteria. Hospital wastewater should be disinfected before it is released into the sewage system [3]. Therefore, the characterization of isolates found in hospital wastewater and the determination of their antibiotic resistance patterns could be valuable in tracing the origins and determining the persistence of the bacteria associated with hospital acquired infections.

This study will help to reduce and eliminate the burden of antibiotic resistance. It was conducted on hospital wastewater samples to determine the prevalence of bacterial pathogens in it and also to isolate multidrug-resistant (MDR) bacterial strains. Furthermore, the authors also aimed to study the antimicrobial pattern of bacterial strains against fourteen different antibiotics.

2. Methodology

2.1. Isolation and Characterization

Five wastewater samples were collected from different drainage regions of government hospitals in Multan, Pakistan. Samples 1, 2 and 3 were collected from Government Nishtar hospital and samples 4 and 5 were collected from Government Chaudhry Pervaiz Elahi Institute of Cardiology, Multan, Pakistan. The samples of wastewater were collected from the open flowing area across the hospital environment under sterilized conditions. Their physiological conditions, that is, temperature and pH were recorded. Each sample was collected in a sterilized glass bottle and immediately transported to the laboratory. The study was approved by the departmental ethical committee. Isolated strains were characterized, both morphologically and biochemically. Gram's staining and endospore staining was performed [7]. They were biochemically identified through Bergey's Manual of Determinative Bacteriology [8].

2.2. Biological Screening

Bacterial test strains were cultured and their antibiotic sensitivity was determined.

2.2.1 Preparation of Bacterial Test Strain

Muller Hinton agar plates were prepared to analyze the antimicrobial activity of bacterial strains [9]. Cultures of bacteria were grown in 3-4 ml sterilized nutrient broth and incubated at 37°C. The turbidity of bacterial broths was adjusted at 0.5 McFarland standard by adjusting the optical density between 0.08 - 0.1 nm at 625 nm. The uninoculated broth was used as blank.

2.2.2 Antibiotics Used

The standard method of disk diffusion was used to determine the antibiotic sensitivity of bacterial isolates [10]. A total of 14 antibiotic discs of standard concentration were used including ampicillin (10 µg), clindamycin (2 µg), ciprofloxacin (5 µg), erythromycin (5 µg), fusidic acid (10 µg), gentamycin (10 µg), linezolid (30 µg), oxacillin (1 µg), quinopristin (15 µg), streptomycin (10 µg), tetracycline (30 µg), trimethoprim (1.25 µg), and vancomycin (30 µg).

2.3 Antibiotic Sensitivity

Kirby-Bauer disc diffusion method was used to check the sensitivity of antibiotics. The area around the disc where bacteria showed no or insufficient growth to be visible was referred to as the zone of inhibition. The cultures resistant to antibiotics showed bacterial growth around the disc, while the cultures sensitive to antibiotics showed no growth around the disc. The results were observed in the form of the zone of inhibition [10].

3. Results

3.1. Isolation

Water samples were collected from different regions of Government Nishtar hospital and Government Chaudhry Pervaiz Elahi Institute of Cardiology, Multan, Pakistan. The pH range was 7-8.1 and the temperature range was 15-35°C. An inoculum of 75 µl from dilutions 10^{-1}, $10^{-3,}$ and 10^{-5} was spread on nutrient agar and incubated at 37°C for 24 hours. A total of 45 strains were isolated and their CFU/ml was determined. It was found that 25 bacterial isolates belonged to *Bacillus* species, 15 bacterial strains belonged to *Staphylococcus* and Micrococcus species, and 4 bacterial strains belonged to *Streptococcus* species. Moreover, *Enterococcus* species were also identified. The majority of bacterial strains belonged to *Bacillus* species.

3.2. Antimicrobial Resistance Pattern

For the screening of antimicrobial resistance, ampicillin (10 µg), chloramphenicol (30 µg), clindamycin (2 µg), ciprofloxacin (5 µg), erythromycin (15 µg), fusidic acid (10 µg), gentamicin (10 µg), linezolid (30 µg), oxacillin (1 µg), quinopristin (15 µg), streptomycin (10 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (5 µg), and vancomycin (30 µg) discs were used (Table 1). The clear zones indicating the antimicrobial pattern around each disc were measured separately in millimeters (Figure 1).

Table 1. Antibiotic Discs Used

Sr. No.	Classes	Name	Conc.	Standard		
				R	I	S
1	Oxazolidinone	Linezolid – LZD	30 µg	≤ 20	21 – 22	≥ 23
2	Glycopeptide	Vancomycin – VA	30 µg	≤ 14	15 – 16	≥ 17
3	Fusidane	Fusidic Acid – FD	10 µg	≤ 17	18-21	≥22
4	Lincomycin	Clindamycin – DA	2 µg	≤ 14	15 – 20	≥ 21
5	Fluoroquinolone	Ciprofloxacin – CIP	5 µg	≤ 15	16 – 20	≥ 21
6	Streptogramins	Quinopristin – QD	15 µg	≤ 15	16 – 18	≥ 19
7	Doxycycline, oxytetracycline, minocycline,	Tetracycline – TE	30 µg	≤ 14	15 – 18	≥ 19
8	Sulphonamides and folic acid inhibitors.	Trimethoprim – W	1.25 µg	≤ 10	11 – 15	≥ 16
9	Penicillin	Oxacillin – OX	1 µg	≤ 10	11 – 12	≥ 13
10	Macrolids	Erythromycin – E	5 µg	≤ 13	14 – 22	≤ 23
11	Aminoglycoside	Gentamycin – CN	10 µg	≤ 12	13 – 14	≥ 15
12	Penicillin	Ampicillin – AM	10 µg	≤11	12-13	≥14
13	Amphenicol.	Chloramphenicol – C	30 µg	≤12	13-17	≥18
14	Folic acid inhibitor. Long & short-lasting sulphonamide.	Sulphonamide – S	30 µg	≤14	15-20	≥21

Key: R = resistant, I = intermediate, and S = sensitive

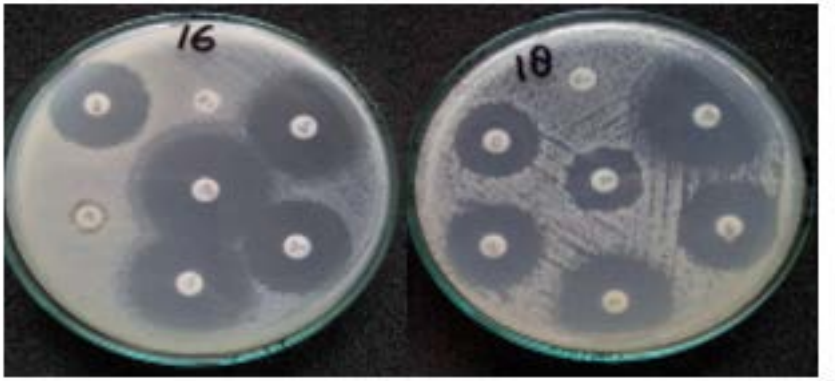

Fig 1. Antimicrobial activity of bacterial isolates

Table 2. Zone of Inhibition

Strain ID	QD	CN	TE	W	OX	E	C	LZD	VA	FD	DA	CIP	S	AM
S1/1	20	20	**9**	15	**8**	15	30	28	15	**17**	20	20	30	19
S1/2	-	21	**9**	14	-	-	-	-	-	-	-	23	28	24
S1/3	20	20	15	-	20	20	29	27	20	**17**	18	25	33	21
S1/4	**14**	23	-	-	-	**13**	25	-	19	**6**	-	25	24	13
S1/5	-	16	**13**	-	-	20	26	-	**13**	**15**	-	25	23	15
S1/6	23	21	18	-	**10**	**12**	21	40	-	-	35	26	28	**10**
S1/7	16	19	**9**	-	-	17	30	-	**12**	20	-	26	27	30
S1/8	35	25	22	15	12	29	33	35	24	29	33	25	36	27
S1/9	23	22	19	-	25	**8**	32	30	21	18	17	28	30	12
S1/10	23	23	22	-	-	**10**	21	34	22	18	20	29	31	14
S2/11	24	25	**10**	23	17	28	27	30	21	27	22	25	32	**10**
S2/12	20	23	**10**	-	-	18	24	26	16	**15**	25	21	29	**9**
S2/13	26	25	16	24	14	**13**	30	27	19	22	21	25	36	**11**
S2/14	26	28	24	-	-	32	33	38	22	23	30	28	37	13
S2/15	25	26	23	-	21	40	31	44	24	30	26	31	33	30
S2/16	22	25	19	-	-	30	26	33	19	19	30	27	30	**10**
S2/17	22	25	21	-	-	33	29	31	18	19	30	27	30	12
S2/18	21	24	20	-	-	30	26	35	20	18	29	27	28	14
S3/19	20	23	18	-	-	30	24	31	18	18	31	29	27	13
S3/20	21	23	**14**	-	-	23	27	34	18	20	27	25	28	13

Strain ID	QD	CN	TE	W	OX	E	C	LZD	VA	FD	DA	CIP	S	AM
S3/21	21	23	**12**	**8**	**8**	23	21	35	15	**16**	25	25	27	12
S3/22	20	14	-	-	-	25	16	-	21	18	-	21	18	**9**
S3/23	21	23	21	-	12	23	28	43	23	**12**	18	33	29	38
S3/24	21	24	21	-	-	34	29	36	19	**17**	28	27	30	**11**
S3/25	19	34	16	-	11	-	30	40	27	18	20	35	35	21
S3/26	18	23	18	-	-	19	24	33	**14**	**16**	28	25	28	**10**
S3/27	19	21	**14**	-	**10**	20	24	30	16	**17**	23	21	25	**10**
S3/28	19	21	**12**	-	-	18	22	30	15	**14**	25	23	25	**10**
S4/29	19	25	18	-	-	24	23	35	16	**16**	21	27	29	13
S4/30	19	22	**14**	-	-	18	28	33	20	**13**	24	26	27	**11**
S4/31	20	21	15	-	-	18	25	30	16	**12**	15	25	28	12
S4/32	21	22	**11**	-	-	17	26	28	**14**	**11**	**14**	26	25	13
S4/33	19	23	**13**	-	**9**	18	23	30	16	**16**	16	30	24	**11**
S4/34	18	21	17	-	-	17	22	31	17	**15**	18	29	25	**10**
S4/35	19	24	18	-	-	18	24	28	16	**17**	21	25	27	**10**

Key: QD; quinopristin, CN; gentamycin, TE; tetracycline, W; trimethoprim, OX; oxacillin, E; erythromycin, C; chloramphenicol, LZD; linezolid, VA; vancomycin, FD; fusidic acid, DA; clindamycin, CIP; ciprofloxacin, S; sulphonamide and AM; ampicillin. Bold numbers show resistant strains against specific antibiotics. Minus sign show resistance against antibiotics.

It was observed that *Bacillus* spp. (strain S1/2) showed maximum resistance against 9 antibiotics including chloramphenicol, clindamycin, erythromycin, fusidic acid, linezolid, oxacillin, quinopristin, tetracycline, and vancomycin. Four strains (S1/2, S1/8, S2/11, and S2/13) showed sensitivity to trimethoprim. Their zones of inhibition were 14 mm, 15 mm, 23 mm, and 24 mm, respectively. However, all other strains showed resistance against the said antibiotic. All strains were sensitive to the antibiotic ciprofloxacin, while no strains showed resistance against it. All isolates were sensitive to antibiotic chloramphenicol except one strain (S1/2), which showed resistance against this antibiotic. One strain (S3/22) showed intermediate resistance to it. Three strains (S1/2, S1/4, and S1/5) showed resistance against the antibiotic quinopristin, while all other strains showed sensitivity towards it. All strains were sensitive to the antibiotic linezolid except five strains (S1/2, S1/4, S1/5, S1/7, and S3/22). These five strains showed resistance against it.

All bacterial isolates were sensitive to ampicillin except fourteen strains, which showed resistance against this antibiotic. All bacterial isolates were also sensitive to the antibiotic sulphonamide and no strain showed resistance against it. Similarly, all bacterial isolates proved to be susceptible to the antibiotic gentamycin. Moreover, 15 strains (S1/1, S1/2, S1/4, S1/5, S1/7, S2/11, S2/12, S3/20, S3/21, S3/22, S3/27, S3/28, S4/30, S4/32 and S4/33) were resistant to the antibiotic tetracycline, while other

strains showed sensitivity towards it (Table 2). Bacterial strains which were resistant to more than one class of antibiotics were classified as multidrug resistance bacteria

3.3. Multidrug-Resistance Frequency among Samples

The maximum number of MDR bacterial strains were isolated from Nishtar hospital wastewater samples (1, 2 and 3). Strains isolated from samples 1 and 4 showed 90% frequency of MDR strains, while all the isolates (100%) were MDR from samples 2 and 3. On the contrary, isolates from sample 5 showed no MDR strains. *Bacillus* species, *Staphylococcus* species, *Streptococcus* species, and *Micrococcus* species were predominantly present in all samples (Table 3).

Table 3: Antibiotic-resistant Bacterial Species

Sample	No. of Isolates	Isolated Species	No. of Strains Resistant against Antibiotics
1	10	1/10 *Bacillus* spp.	9/10 (90%)
		5/10 *Staphylococcus* spp.	
		3/10 M*icrococcus* spp.	
		1/10 S*treptococcus* spp.	
2	8	6/8 *Bacillus* spp.	8/8 (100%)
		2/8 S*treptococcus* spp.	
3	10	6/10 *Bacillus* spp.	10/10 (100%)
		2/10 *Staphylococcus aureus*	
		1/10 *Staphylococcus* spp.	
		1/10 *Streptococcus* spp.	
4	9	6/9 *Bacillus* spp.	7/8 (90%)
		2/9 *Staphylococcus* spp.	
5	8	6/8 *Bacillus* spp.	_
		2/8 *Micrococcus* spp.	

Negative sign shows that no species is resistant to antibiotics

4. Discussion

In the current study, isolated strains belonged to *Bacillus* spp., *Micrococcus* spp., *Streptococcus* spp., *Staphylococcus* spp., and *Enterococcus* spp. The presence of Gram-negative bacteria in hospital wastewater has been reported previously. Moreover, *Bacillus* spp., *Streptococci* spp.,

and *Staphylococci* spp. have been commonly reported previously as well [4]. MDR bacterial strains identified in this study were *Bacillus* spp., *Micrococcus* spp., *Staphylococcus* spp., *Streptococcus* spp., and *Enterococcus* spp. High MDR was observed in Gram-positive rods, such as *Bacillus* spp. and in Gram-positive cocci, such as *Streptococcus* spp. and *Staphylococcus* spp. Furthermore, the incidence of MDR was determined to be higher than previously reported by Panday et al [10]. The findings of the current study are contradictory to the findings of the study of Aggarwal et al., in which *Pseudomonas* spp. were isolated and found resistant to five antibiotics. On the contrary, no *Pseudomonas* spp. were identified in the current study. Additionally, the authors also observed that ampicillin resistance was found in the maximum number of isolates [11].

Hospitals have higher numbers of antibiotic residues and this explains the presence of MDR bacteria. Hospital wastewater carries 25% more antibiotics than community wastewater [12]. Therefore, sewage water should be processed to minimize the number of bacteria in it [10-13]. Antimicrobial resistance is a global threat that negatively affects the health and economy of a country. The presence of resistant bacterial isolates in hospital waste demands the availability of effectual treatment plants to treat the waste before it is disposed of into waste lines. Besides taking these steps, public awareness is crucial to avoid MDR bacteria at the personal and community level [14, 15]. Several studies have revealed that resistant strains are more abundant in areas close to cities than in far flung rural areas, indicating anthropogenic contamination. Similar results were obtained in this study. No significant difference in resistant strains was found in all samples as these were all hospital samples. However, studies based on a different sampling source showed a significant difference in the prevalence of antibiotic-resistant bacteria. Other cities in Pakistan have also reported significantly higher rates of water contamination with coliforms, toxic metals, and pesticides [16, 17]. Environmental contaminants are associated with poorly managed social development. Rapid migration from rural to urban areas, uncontrolled expansion of cities, and continuous disappearance of green land coupled with unfit biowaste management practices currently accelerate the spread of resistant microorganisms in underground reservoirs, that is, water and soil [17]. A relatively new approach known as sewage epidemiology approach should be adopted along with the wastewater epidemiology approach to identify infected areas. There is an urgent need for the optimization of waste collection infrastructure and water distribution systems. The biowaste management sector should work in coordination with the local research and health communities to prevent all possible risks [7, 14, 18].

Conclusion

The current study showed that large numbers of MDR bacteria are found in hospital wastewater and most of the isolates identified were Gram-positive rods. A total of 14 antibiotics including ampicillin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, fusidic acid, gentamycin, linezolid, oxacillin, quinopristin, sulphonamide, tetracycline, trimethoprim, and vancomycin were used against the isolates, all of which except isolate 8 showed resistance against one or more classes of antibiotics. All the isolated strains from samples 2 and 3 (100%) and

most strains from samples 1 and 4 (90%) were MDR. Most of the *Bacillus* spp., *Micrococcus* spp., *Staphylococcus* spp., and *Streptococcus* spp. were present in all hospital wastewater samples.

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Conflict of Interest

The authors declare no conflict of interest.

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