Molecular investigation of antibiotic resistant bacterial strains isolated from wastewater streams in Pakistan

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Abstract

Antibiotic resistance is a global public health issue and it is even more daunting in developing countries. The main objective of present study was to investigate molecular responses of antibiotic-resistant bacteria. The 48 bacterial strains, which were previously isolated and identified were subjected to disc diffusion and MIC (minimum inhibitory concentration) determination, followed by investigating the production of the three beta-lactamases (ESBLs (Extended-spectrum Beta-lactamases), MBLs (Metallo Beta-lactamases), AmpCs) and exploring prevalence of the two antibiotic-resistant genes (ARGs); *blaTEM* and *qnrS*. Higher MIC values were observed for penicillin(s) than that for fluoroquinolones (ampicillin > amoxicillin > ofloxacin > ciprofloxacin > levofloxacin). Resistance rates were high (58–89%) for all of the tested beta-lactams. Among the tested strains, 5 were ESBL producers (4 *Aeromonas* spp. and 1 *Escherichia* sp.), 2 were MBL producers (1 *Stenotrophomonas* sp. and 1 *Citrobacter* sp.) and 3 were AmpC producers (2 *Pseudomonas* spp. and 1 *Morganella* sp.). The ARGs *qnrS2* and *blaTEM* were detected in *Aeromonas* spp. and *Escherichia* sp. The results highlighted the role of *Aeromonas* as a vector. The study reports bacteria of multidrug resistance nature in the wastewater environment of Pakistan, which harbor ARGs of clinical relevance and could present a public health concern.

Keywords Antibiotic resistance · Antibiotic resistance genes · Beta-lactamases · Resistant bacteria · Wastewater

Introduction

Overuse and misuse of antibiotics by humans, veterinary field, aquaculture and agriculture are causing development and spread of antibiotic resistance in environment throughout the world (French 2005; Saima et al. 2020). Pakistan

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stands third among the lower middle-income countries for antibiotic consumption. Over a period of 15 years (2000–2015), there has been 68% rise in antibiotic consumption in Pakistan (Klein et al. 2015). Apart from chemical pollution (Mukhtar et al. 2020), the presence of antibiotics in the environment also causes development of ARGs (Arshad and Zafar 2020), which shade health impacts and can transfer antibiotic resistance to humans and animals through direct or indirect contact (Osińska et al. 2016). The ARGs and associated genes, which function in acquisition and dissemination of antibiotic resistance have been reported in clinical and environmental bacteria (Pormohammad et al. 2019). There is growing evidence that clinical antibiotic resistance is intimately associated with environmental bacteria and ARGs. In fact, it is believed that ARGs now present in the pathogenic bacteria have evolved from microbes, which naturally produce antibiotics or their natural competitors (Aminov et al. 2007; Arshad and Zafar 2020). They may evolve due to recombinatorial events under the influence of anthropogenic impact in the aquatic environment (Baquero et al. 2008), which may result in development of novel resistance mechanisms, thus making it hard to deal with while antibiotics continue to lose their effectiveness.

Even though antibiotic resistance is a global health issue, it has been majorly overlooked in the aquatic environment (Khan et al. 2013), especially in developing countries. A study reported that typhoid-causing *Salmonella* are gaining resistance against second line antibiotics, which mostly include beta-lactams and fluoroquinolones. This has resulted in development of multidrug-resistant and extensively drug-resistant *Salmonella* typhi in Pakistan (Azhar et al. 2019). Only a few studies have been conducted, which report about the prevalence of ARGs in aquatic environment of Pakistan. Shah et al. (2012) reported that the ESBL-encoding gene *blaTEM* was the most prevalent ARG in bacterial DNA directly isolated from fish, fish pond water and sediments of Pakistan and Tanzania. Similarly, DNA isolated from sediments of the aquatic environment of six main rivers of Pakistan had high levels of antibiotics and ARGs (*sulI, dfrAI*) and other associated genes (*intl1*), which indicates that the rivers may act as reservoirs of ARGs and disseminate them (Khan et al. 2013). Despite the obvious importance of water and wastewater in emergence and spread of antibiotic resistance and its determinants (Djenadi et al. 2018; Khan et al. 2013; Haller et al. 2018; Oliveira et al. 2017), it remains poorly investigated in Pakistan.

The objectives of this study were to (1) investigate antibiotic-resistant bacterial (ARBs) strains previously isolated from various wastewater streams for MICs determinations against five broad-spectrum antibiotics, beta-lactamase (A-C) characterization, and (2) PCR detection of the ARGs *blaTEM* and *qnrS* to observe their possible co-existence. The plasmids that carry ESBL genes (blaTEM, blaCTX-M)

often lodge resistance determinants for fluoroquinolones and other antibiotics (Carattoli 2011) and, thus, present a risk of transfer of resistance to more than one antibiotic at the same time. This work has contributed significant information on rarely studied antibiotic-resistant bacteria isolated from wastewater streams for MIC determination, beta-lactamase characterization and ARGs detection in Pakistan.

Materials and methods

Bacterial strains

The bacterial strains (48) used in the study were previously isolated from wastewater streams of Islamabad (33.67 N, 73.06 E) (33.70 N, 73.04 E) in Faisalabad (31.38 N, 73.06 E) and Rawalpindi (33.63 N, 73.04 E). All of the wastewater streams were near hospitals and small health clinics. The samples were diluted by tenfold and dominant colonies were selected on nutrient agar (data not shown here) and stored at – 70 °C in 70% glycerol stocks. The strains were identified by sequencing of 16 s rRNA gene. All of the identified strains were Gram-negative except *Bacillus* sp., which was Gram-positive. The bacterial population consisted of *Pseudomonas* spp., *Aeromonas* spp., *Acinetobacter* spp., *Escherichia* spp., *Proteus* sp., *Morganella* sp., *Bacillus* sp., and *Citrobacter* sp., *Shigella* sp., *Comamonas* spp. and *Stenotrophomonas* sp. All of the following experiments were repeated three times. The details of the strains are summarized in Table 1.

MICs (minimum inhibitory concentrations)

The antibiotics chosen for testing in this study are broad-spectrum and are commonly used drugs not only in Pakistan but also the whole world (Wilke et al. 2005; CDDEP 2017) as they are considered a cure all by consumers, which is one of the reasons for their increased consumption. MICs of AMP (ampicillin), AMX (amoxicillin), CIP (ciprofloxacin), OFX (ofloxacin) and LEV (levofloxacin) (Sigma Aldrich) of the strains were determined with agar-dilution method. A range of concentrations (256–0.125 µg mL^{-1}) recommended by CLSI (Clinical and Laboratory Standards Institute) (2012) were tested.

Disc diffusion test

Strains were further screened by disc diffusion test for resistance against CTX (cefotaxime, 30 µg), CAZ (ceftazidime, 30 µg), CPD (cefpodoxime, 10 µg), IPM (imipenem, 10 µg) and FOX (cefoxitin, 30 µg) (CLSI 2018). The strains were incubated overnight in tryptic soy agar (Oxoid) and adjusted to a turbidity of 0.5 McFarland standard in 0.85% saline

Table 1 Bacterial genera with their strain IDs along with their respective locations form where they were isolated and antibiogram of the isolated strains

Strain ID	Location		Genus	Antibiogram				
				CTX	CAZ	CPD	IPM	FOX
NCCP-1704	33 38 09.03 N	73 02 03.06 E	*Aeromonas* spp.	R	I	R	S	R
NCCP-1714	–			I	I	R	I	R
NCCP-1724	–			I	R	R	I	R
NCCP-1757	31 22 48.39 N	73 04 20.19 E		R	R	R	R	R
NCCP-1751	–			R	R	R	R	R
NCCP-1748	–			R	R	R	I	R
NCCP-1749	–			R	R	R	I	R
NCCP-1769	–			I	R	R	I	R
NCCP-1728	33 38 09.03 N	73 02 03.06 E	*Escherichia* spp.	R	R	R	R	R
NCCP-1731	–			R	R	R	R	R
NCCP-1753	31 22 48.39 N	73 04 20.19 E		R	R	R	R	R
NCCP-1754	–			R	R	R	R	R
NCCP-1802	33 42 10.82 N	73 02 45.34 E		R	I	R	R	R
NCCP-1717	33 38 09.03 N	73 02 03.06 E	*Acinetobacter* spp.	R	R	N.D	R	N.D
NCCP-1718	–			R	R	N.D	R	N.D
NCCP-1720	–			R	R	N.D	I	N.D
NCCP-1721	–			R	R	N.D	I	N.D
NCCP-1723	–			R	R	N.D	R	N.D
NCCP-1813	33 42 10.82 N	73 02 45.34 E		R	R	N.D	S	N.D
NCCP-1758	31 22 48.39 N	73 04 20.19 E		R	R	N.D	R	N.D
NCCP-1760	–			R	R	N.D	S	N.D
NCCP-1761	–			R	R	N.D	I	N.D
NCCP-1762	–			R	R	N.D	I	N.D
NCCP-1763	–			R	R	N.D	I	N.D
NCCP-1744	–		*Citrobacter* spp.	R	R	R	R	R
NCCP-1750	–			R	R	R	R	R
NCCP-1742	33 38 09.03 N	73 02 03.06 E	*Comamonas* spp.	R	R	N.D	R	N.D
NCCP-1786	33 40 30.75 N	73 04 05.31 E		R	R	N.D	S	N.D
NCCP-1771	–		*Proteus* sp.	R	R	R	R	R
NCCP-1830	–		*Shigella* sp.	R	R	R	R	R
NCCP-1756	31 22 48.39 N	73 04 20.19 E	*Bacillus* sp.	S	S	S	S	S
NCCP-1829	33 40 30.75 N	73 04 05.31 E	*Stenotrophomonas* sp.	R	R	R	R	R
NCCP-1791	–		*Morganella* sp.	R	R	R	R	R
NCCP-1709	33 38 09.03 N	73 02 03.06 E	*Pseudomonas* spp.	R	R	N.D	R	R
NCCP-1712	–			R	R	N.D	R	R
NCCP-1713	–			R	R	N.D	R	R
NCCP-1773	33 40 30.75 N	73 04 05.31 E		R	R	N.D	R	R
NCCP-1780	–			R	R	N.D	R	R
NCCP-1781	–			R	R	N.D	R	R
NCCP-1782	–			R	R	N.D	R	R
NCCP-1783	–			R	R	N.D	I	R
NCCP-1784	–			R	R	N.D	I	R
NCCP-1787	–			R	R	N.D	I	R
NCCP-1788	–			R	R	N.D	R	R
NCCP-1801	33 42 10.82 N	73 02 45.34 E		R	R	N.D	R	R
NCCP-1803	–			R	R	N.D	I	I
NCCP-1805	–			R	R	N.D	R	R

Table 1 (continued)

Strain ID	Location	Genus	Antibiogram				
			CTX	CAZ	CPD	IPM	FOX
NCCP-1808	–		I	I	N.D	S	I
Total strains (48)		Resistant strains	42	43	19	28	32
		Resistant strains (percentage)	87.5	89.5	*	58.3	*

The dash (–) here indicates that the location is the same as above, while the boxes with N.D. mean that the susceptibility test wasn't conducted for these strains due to lack of inhibition zone interpretation criteria, that is why the total resistant strains in percentage for those antibiotics were not calculated, as indicated by the asterisk (*)

CPD cefpodoxime, *CTX* cefotaxime, *CAZ* ceftazidime, *IPM* imipenem, *FOX* cefoxitin, *R* resistant, *I* intermediate, *S* susceptible, *ND* not determined

(NaCl) and streaked on Mueller Hinton Agar (MHA). The MHA plates were then incubated for 18–20 h at 35 °C. The quality control strain *Escherichia coli* (ATCC 25922) was used. All the discs were purchased from Bioanalyse. The inhibition zones were interpreted according to the Table 2.

Beta-lactamases

ESBL detection was done only in *Aeromonas* spp. and *Enterobacteriaceae* with double-disc synergy (DDST) and combination disc test (CDT), which had been previously done by Rameshkumar et al. (2016). In CDT, ESBL production was confirmed using CAZ and CTX alone and in combination with clavulanic acid (10 + 30 μg) in combination disc assay (CLSI 2018). Inhibition zone difference of ≥ 5 mm between CTX and CAZ alone and in combination with clavulanic acid was considered to be positive for ESBL production. In DDST, CAZ and CTX were placed on both sides of amoxicillin + clavulanate (AMC, 20 + 10 μg) disc

(Jarlier et al. 1988). Appearance of synergistic zone between CAZ or CTX and AMC was considered to be positive for ESBL production.

The method described by Yong et al. (2002) was used for detection of MBLs only in *Stenotrophomonas* sp. and *Citrobacter* sp. which had also been used by Daef et al. (2017) and Rizvi et al. (2009). In this confirmation test, imipenem was used alone and in combination with EDTA (10 + 750 μg). Zone difference of ≥ 6 mm was considered to be positive for MBL production.

AmpC production confirmation was done for *Pseudomonas* spp. and *Morganella* sp. only with the disc-approximation method (Mathur et al. 2014) as had also been previously done by Oliveira et al. (2017). In confirmation test, IPM, CAZ, AMC and FOX were used. The Zone flattening of CAZ towards any of the substrate disc was considered to be positive for AmpC production.

Table 2 The inhibition zone interpretation criteria used for each genus

Genus	CTX	CAZ	CPD	IMP	FOX
Aeromonas spp. *Escherichia* spp. *Citrobacter* spp. *Proteus* sp. *Shigella* sp. *Morganella* sp. *Stenotrophomonas* sp.	*Enterobacteriaceae* (CLSI 2018)	*Enterobacteriaceae* (CLSI 2018)	*Enterobacteriaceae* (CLSI 2018)	*Enterobacteriaceae* (CLSI 2018)	*Enterobacteriaceae* (CLSI 2018)
Acinetobacter spp.	CLSI (2018)	CLSI (2018)	N.D	CLSI (2018)	N.D
Pseudomonas spp.	Andrews and Howe (2011)	Andrews and Howe (2011)	N.D	Andrews and Howe (2011)	Upadhyay et al. (2010)
Comamonas spp.	*Pseudomonas* spp.	*Pseudomonas* spp.	N.D	*Pseudomonas* spp.	N.D
Bacillus sp.	Predicted from FOX result	Predicted from FOX result	Predicted from FOX result	Predicted from FOX result	*Staphylococcus* spp. (CLSI, 2018)

Although *Stenotrophomonas* and *Aeromonas* spp. are not *Enterobacteriaceae*, the same inhibition zone interpretation criterion has been used for it because, it has been used previously in published literature (Harmon et al. 2018; Rameshkumar et al. 2016). The empty boxes indicate that susceptibility tests have not been conducted for those genera. As CLSI doesn't provide inhibition zone breakpoints for *Pseudomonas* spp., they have been interpreted according to published literature (Andrews and Howe 2011; Upadhyay et al. 2010)

PCR for detection of *blaTEM* and *qnrS*

The ARG *blaTEM*, is a TEM enzyme-encoding gene (hydrolyzed beta-lactams). They are of high significance with regard to prevalence, distribution, and diversity. Wherein, *qnrS* is qnr protein-encoding gene, which is commonly associated with ESBLs (TEM, CTX-M). Rapid preparation of template DNA was done by heating (95 °C for 10 min) a single pure colony in PCR-grade water followed by a centrifugation step (Dallenne et al. 2010). The supernatant of 2 µL was subjected to PCR in a 50 µL mixture containing 25 µL PCR mix (Thermo Fisher), 2 µL (0.4 pmol µL^{-1}) of both forward and reverse primers (Table 3). The cycling conditions were: Initial denaturation at 94 °C for 7 min; 35 cycles of denaturation, annealing and amplification at 94 °C for 40 s, 64 °C (*qnrS*), 56 °C (*blaTEM*) for 40 s and 72 °C for 1 min, respectively, and final extension for 7 min at 72 °C. Finally, the PCR product was run on agarose gel (1%) stained with ethidium bromide at 100 V for 1 h and visualized with UV transilluminator, 100 bps ladder (Thermo Fisher) was used as size marker. After gel confirmation, the PCR product was sequenced by Macrogen (Korea). The obtained sequences were run on BLAST algorithm for checking homology. The sequences have been submitted into Genbank. ExPASy was used for translation of the nucleotides to amino acid sequences.

Results

MICs (minimum inhibitory concentrations)

The detailed results related to MICs are presented in Table 4. MICs values were of the following order: ampicillin > amoxicillin > ofloxacin > ciprofloxacin > levofloxacin (Table 4). About 31% of the strains had the highest tested MIC of ampicillin, which in this case was 256 µg mL^{-1}. Among them, 47% were *Aeromonas* spp. followed by 13% *Escherichia* spp., 13% *Citrobacter* spp., and 13% *Pseudomonas* spp. Similar results were observed for amoxicillin with 28.8% of the strains having the highest tested MIC, among which 37% of the strains were *Aeromonas* spp., 19% *Pseudomonas* spp., followed by 12% *Escherichia* spp. and 12% *Citrobacter* spp.

Among the fluoroquinolones, 29% of the strains had high MICs (256 and 128 µg mL^{-1}) of ciprofloxacin and ofloxacin together. Among them, 28% were *Aeromonas* spp., 28% were *Escherichia* spp., followed by 21% *Citrobacter* spp. and 7% *Pseudomonas* spp. MICs for levofloxacin were generally low with the only one *Escherichia* sp. and one *Acinetobacter* sp. showing the highest MIC, which was 8 µg mL^{-1}.

Disc diffusion

The strains were highly resistant to third-generation cephalosporins. The bacterial resistance towards CAZ, CTX and IPM was 89% > 87% > 58%, respectively. Percentage resistance to CPD and FOX was not calculated because their susceptibility tests were not conducted for *Pseudomonas* spp., *Acinetobacter spp.* and *Comamonas* spp. The genus *Aeromonas* exhibited high-resistance rates to all of the tested antibiotics. Whereas, the most effective antibiotic among the tested ones was observed to be imipenem. The detailed data can be observed in Table 1.

Beta-lactamases

In total, there were 21% (10 out of 48) beta-lactamase producers, among which 5 strains were ESBL producers, 2 strains were MBL producers and only 3 strains were AmpC producers. The AmpC production by the two strains could be inducible. All of the ESBL producers were from the genus *Aeromonas* except for one strain, which was *Escherichia* sp. The MBL producers consisted of one *Stenotrophomnas* sp. and one *Citrobacter* sp. Two of the AmpC producers were from the genus *Pseudomonas*, while only one was from the genus *Morganella.* The pictorial evidence is shown in Fig. 1 and further details are given in Table 5.

ARGs

The genes *qnrS2 and blaTEM* were up to 100% match to sequences detected in *Escherichia coli* and *Klebsiella pneumoniae* (Accession no. CP053728, CP054237, MH684532) isolated from clinical samples. The sequences of *qnrS2* and *blaTEM* were deposited in Genbank under the accession numbers MK359204, MN218676 and MN218677. The gel image of bands is shown in Fig. 2. Blast search and alignment of all the three sequences are presented in supplementary material (Figures S1, S2 and S3).

Table 3 Primers used in this study with their base length and product size

Primer	Sequence (5′ → 3′)	Length (bases)	Product (bases)	References
qnrS-F	GCAAGTTCATTGAACAGGGT	20	739	Cattoir et al. (2008)
qnrS-R	TCTAAACCGTCGAGTTCGGCG	21		
bla$_{TEM}$-F	CATTTCCGTGTCGCCCTTATTC	22	329	Dallenne et al. (2010)
bla$_{TEM}$-R	CGTTCATCCATAGTTGCCTGAC	22		

Table 4 Minimal inhibitory concentrations (MICs, μg mL^{-1}) of the bacterial strains for five antibiotics

Genus (total isolates)	No. of strains (MIC μg mL^{-1})				
	AMP	AMX	OFX	CIP	LEV
Acinetobacter spp. (11)	1 (0.125)	1 (0.25)	2 (0.125)	1 (0.125)	7 (0.125)
	1 (0.5)	2 (2)	1 (0.25)	4 (0.25)	2 (0.25)
	2 (2)	2 (4)	7 (2)	3 (1)	1 (0.5)
	1 (8)	2 (8)	1 (8)	2 (2)	1 (8)
	4 (32)	1 (16)		1 (8)	
	1 (64)	1 (32)			
	1 (256)	1 (64)			
		2 (128)			
Aeromonas spp. (8)	1 (64)	1 (8)	1 (0.125)	1 (0.25)	5 (0.125)
	7 (256)	1 (64)	1 (1)	1 (0.5)	1 (0.25)
		6 (256)	1 (4)	3 (2)	2 (4)
			1 (8)	1 (64)	
			2 (16)	2 (128)	
			1 (128)		
			1 (256)		
Citrobacter spp. (2)	2 (256)	2 (256)	1 (256)	2 (256)	2 (4)
			1 (16)		
Comamonas spp. (2)	2 (32)	1 (32)	1 (0.25)	1 (2)	2 (0.125)
		1 (8)	1 (4)	1 (128)	
Escherichia spp. (5)	2 (32)	1 (0.5)	2 (0.25)	2 (0.25)	2 (0.125)
	1 (64)	1 (32)	3 (256)	1 (2)	2 (4)
	2 (256)	1 (64)		1 (128)	1 (8)
		2 (256)		1 (256)	
Morganella sp. (1)	16	4	0.125	16	0.125
Proteus sp. (1)	32	4	1	0.25	0.125
Pseudomonas spp. (15)	3 (0.25)	2 (0.25)	8 (0.125)	3 (0.125)	13 (0.125)
	1 (0.5)	2 (0.5)	2 (0.25)	1 (0.25)	1 (0.25)
	8 (32)	4 (4)	1 (0.5)	3 (0.5)	1 (4)
	1 (64)	1 (8)	1 (4)	5 (1)	
	2 (256)	1 (16)	1 (16)	2 (2)	
		2 (32)	1 (64)	1 (32)	
		3 (256)	1 (256)		
Shigella sp. (1)	32	2	0.25	1	2
Stenotrophomonas sp. (1)	256	256	0.5	64	0.125
Bacillus sp. (1)	64	32	8	16	2

Outside of bracket, the digits represent the no. of strains, while inside the bracket is minimal inhibitory concentration (MIC) of that strain calculated in μg mL^{-1}

AMP ampicillin, *AMX* amoxicillin, *OFX* ofloxacin, *CIP* ciprofloxacin, *LEV* levofloxacin

Discussion

Antibiotic susceptibility/resistance of bacteria isolated from wastewater streams in Pakistan is rarely studied. There are various reports of typhoid-causing extensively beta-lactam-resistant *Salmonella* (Azhar et al. 2019). Third-generation cephalosporins and fluoroquinolones are drugs now more commonly prescribed in Pakistan (CDDEP 2017) and carbapenems (imipenem) are our last resort antibiotics for some bacterial infections (Djenadi et al. 2018). High resistance to these antibiotics among environmental bacteria is concerning since there are not many other treatment options and their resistance determinants may be plasmid-borne and transferrable. We report that the environmental bacteria isolated, showed high resistance to the third-generation cephalosporins, cephamycin and carbapenem. Environmental bacteria resistant to third-generation cephalosporins, imipenem and
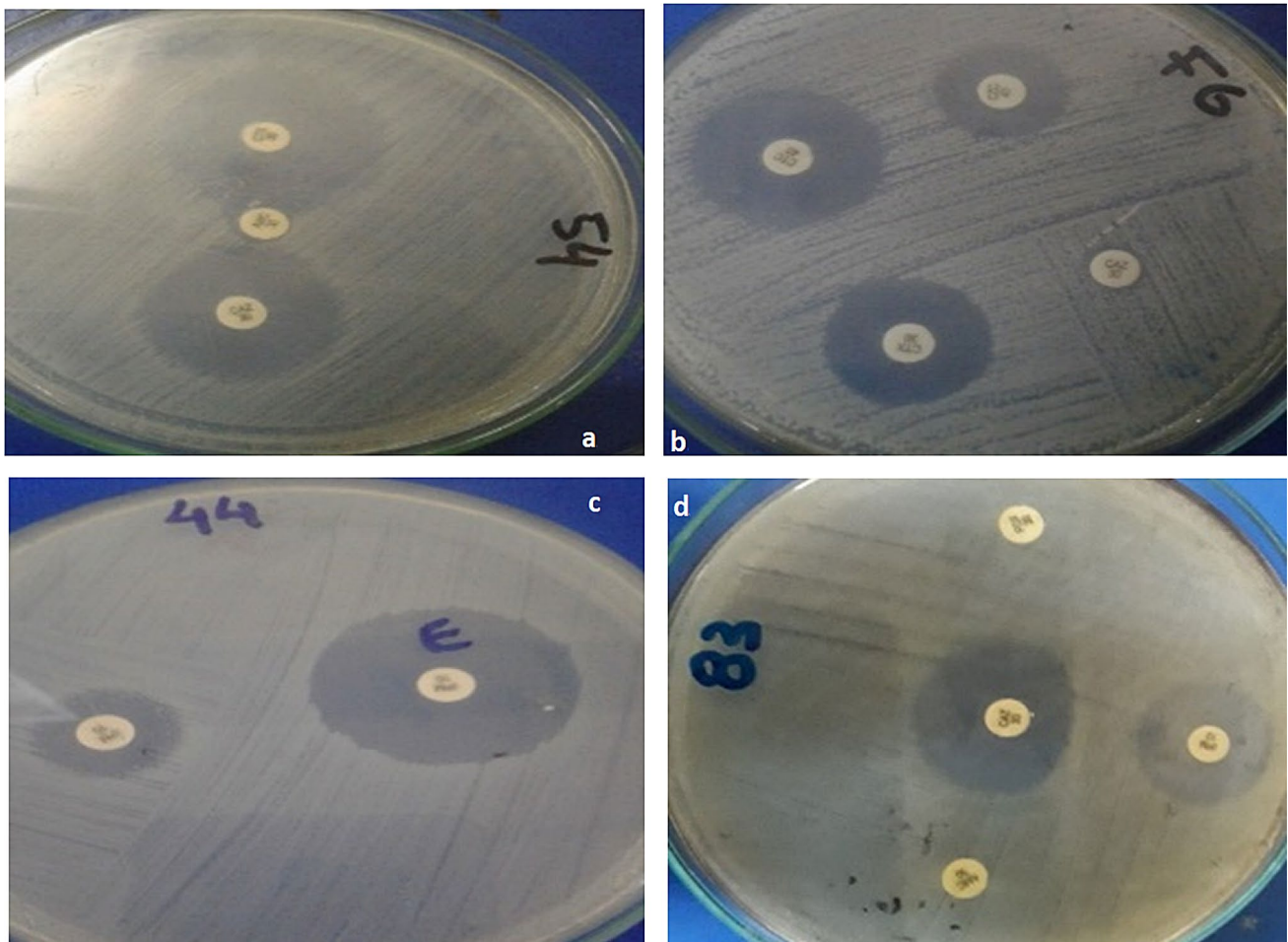
Fig. 1 Beta-lactamase production by resistant bacteria; **a** *Escherichia* sp. (ESBL production confirmed by double disc synergy test) **b** *Aeromonas* sp. (ESBL production confirmed by combination disc test) **c** *Citrobacter* sp. (MBL production confirmed by combination disc test using EDTA) **d** *Pseudomonas* sp. (AmpC production confirmed by disc approximation test)

Table 5 Antibiotic resistance profile of the ARG carrier and beta-lactamase producer bacterial strains

Strain ID	Bacterial strain	Antibiotics resistance Profile	Beta-lactamases			ARGs
			ESBL	MBL	AmpC	
NCCP-1754	*Escherichia* sp.	CTX + CAZ + CPD + FOX + IPM	+			*blaTEM*
NCCP-1751	*Aeromonas* sp.	CTX + CAZ + CPD + IPM + FOX	+			
NCCP-1749	*Aeromonas* sp.	CTX + CAZ + CPD + FOX	+			*qnrS2*
NCCP-1757	*Aeromonas* sp.	CTX + CAZ + CPD + IPM + FOX	+			
NCCP-1769	*Aeromonas* sp.	CAZ + CPD + FOX	+			*blaTEM*
NCCP-1744	*Citrobacter* sp.	CTX + CAZ + CPD + IPM + FOX		+		
NCCP-1829	*Stenetrophomonas* sp.	CTX + CAZ + CPD + IPM + FOX		+		
NCCP-1782	*Pseudomonas* sp.	CTX + CAZ + IPM + FOX			+	
NCCP-1787	*Pseudomonas* sp.	CTX + CAZ + FOX			+	
NCCP-1791	*Morganella* sp.	CTX + CPD + IPM + FOX			+	

The symbol (+) indicates that the strain is a producer of that beta-lactamase

CPD cefpodoxime, *CTX* cefotaxime, *CAZ* ceftazidime, *IPM* imipenem, *FOX* cefoxitin

Fig. 2 PCR amplification; gel image of *blaTEM* and *qnrS2* in comparison with the molecular ladder. Ladder; 100 bps molecular ladder, L2; *qnrS2* identified in *Aeromonas* sp., L6 and L7; *blaTEM* identified in *Escherichia* sp. and *Aeromonas* sp.
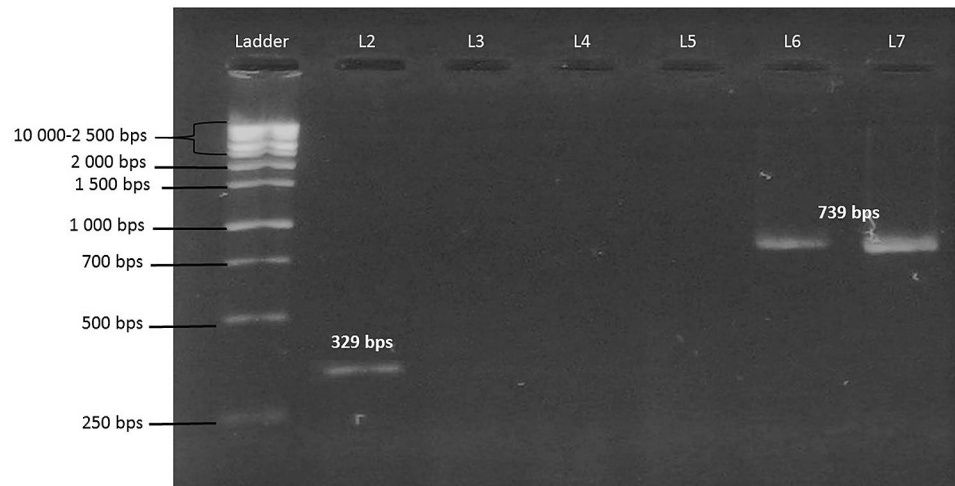
cefoxitin have been reported from Brazil (Oliveira et al. 2017), Algeria (Djenadi et al. 2018), Singapore (Haller et al. 2018) and Thailand (Tansawai et al. 2018). Resistance to third-generation cephalosporins was conferred to production of ESBLs in 5 (*Aeromonas* spp. and *Escherichia* sp.) of the resistant strains. Despite high-resistance rates of *Pseudomonas* spp. and *Acinetobacter* spp. to the third-generation cephalosporins, no ESBL producers were detected among the genera. ESBL production has been reported in *Aeromonas* spp. and *Escherichia* sp. from environmental settings (Tacão et al. 2014). It is well established that beta-lactamases make up one of the major defence mechanisms against beta-lactams (Weldhagen 2004).

High resistance rates (58%) were observed for imipenem. Comparable rates of resistance were also reported by Haller et al. (2018) for bacterial isolates from hospital effluents. Two of the strains (*Stenotrophomnas* sp. and *Citrobacter* sp.) were confirmed for MBL production. MBL production has been previously reported in *Stenotrophomonas* sp. isolated from faeces of a migratory bird (Kenzaka and Tani 2018) and *Citrobacter* sp. isolated from clinical samples (Rizvi et al. 2009).

Among the resistant strains, only 3 were AmpC producers, which were from genus *Pseudomonas* and *Morganella*. The AmpC producing clinical and environmental strains of *Pseudomonas* spp. were reported from Brazil (Oliveira et al. 2017). *Pseudomonas* spp. especially *Pseudomonas aeruginosa* are adept at acquiring antibiotic resistance to various antibiotics by having selective porins, over expressed efflux pump and inducible chromosomal beta-lactamase gene (Hauser 2018). Similarly, the AmpC production has been reported in *Morganella morganii* isolated from food samples in China (Ye et al. 2017). Besides production of beta-lactamases, resistance to beta-lactams is also largely attributed to other resistance mechanisms like loss of porins

(impermeability) and over active efflux systems (Marangon et al. 2004).

High MICs and resistance rates of ampicillin and amoxicillin in Gram-negative environmental bacterial isolates have been reported (Li et al. 2009; Pontes et al. 2009; Sidrach-Cardona et al. 2014; Goni-urriza 2000). In another study, *Acinetobacter* spp. isolated from pharmaceutical plant and hospital wastewater with comparable MICs were also reported (Paiva et al. 2017). Beta-lactams are antibiotics of frequent use; hence, resistance to these is more commonly observed in environmental bacteria (Pontes et al. 2009).

High MICs of ciprofloxacin were reported (Osińska et al. 2016) for some environmental bacteria isolated from discharge of WWTP and river water. The same was observed in our study, where ciprofloxacin MICs as high as 256 µg mL^{-1} were noted for *Aeromonas*. Other studies report results different than ours where no environmental *Aeromonas* strain with MIC higher than 32 µg mL^{-1} was observed (Cattoir et al. 2008; Goni-Urriza 2000). Levofloxacin was noted to be the most effective antibiotic in this study. It inhibited most of the strains at low concentrations (0.0125–8 µg mL^{-1}) compared to the other antibiotics. Similar results for environmental *Enterobacteriaceae* have been reported (Paiva et al. 2017). Most of the strains had low MICs of levofloxacin, even though some of the same isolates had the highest tested MICs (256 µg mL^{-1}) of other fluoroquinolones (ciprofloxacin and ofloxacin). Environmental *Aeromonas* spp. and *Pseudomonas* spp. with low-resistance rates to levofloxacin have been reported (Blasco et al. 2008). Levofloxacin is a relatively newer drug from its class and has not been used extensively in prophylaxis, which might explain the low rates of resistance to this antibiotic (Marangon et al. 2004).

The present study showed that not many strains were beta-lactamase producers (only 10), which makes us think that the high-resistance rates to such multitude of antibiotics (mostly beta-lactams) could be caused by the presence of

other multiple resistance mechanisms as was also reported by Li et al. (2009).

Keeping in view the results of the preliminary tests (MICs, disc diffusion) and importance of the tested antibiotics, it was imperative to investigate the ARGs *blaTEM* and *qnrS*, the latter is considered to be of environmental origin although it confers only low level of resistance to fluoroquinolones. The blast search for qnrS sequences showed them to be highly identical to extracted sequences from *E. coli* and *E. roggenkampii* (Acession no. CP053728, LC545449) found in clinical samples and hospital sewage in China and Japan, respectively. We were able to detect *qnrS2* in only one *Aeromonas* sp., which allows us to think that the genetic machinery responsible for fluoroquinolone resistance in the environmental isolates in this study is much more diverse. Other resistance mechanisms could include, overactive efflux system, alterations in genes encoding enzymes, such as DNA gyrase and topoisomerase IV, and further plasmid-mediated resistance mechanisms (Paiva et al. 2017; Tacão et al. 2014). The PMQR gene, *qnrS2* was detected in ESBL phenotype *Aeromonas* sp. There are several studies reporting qnrS2 in *Aeromonas* spp. from environmental settings (Cattoir et al. 2008). It is not uncommon for ESBL producer to carry resistance genes for other antibiotics like fluoroquinolone and plasmid carrying ESBL-encoding genes often ensnare ARGs encoding resistance to other non-beta-lactam drugs (Cattoir et al. 2008).

The ARG *blaTEM* highly matched extracted sequences from *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella enterica* (Accession no. CP054237, MH684532, CP053703), which were isolated from clinical samples in Texas, India. In our study, only two strains from genera *Aeromonas* sp. and *Escherichia* sp. carried the ARG *blaTEM,* which was unexpected since high MICs (32–256 µg mL^{-1}) of ampicillin and amoxicillin and high-resistance rates to the third-generation cephalosporin were observed for many strains. It is possible that the ESBL producers in our study may have harbored other ESBL-encoding genes (such as, CTX-M, SHV) but as they were not investigated, we were not able to deduce anything. A similar case was presented in another study, where highly beta-lactam-resistant bacterial strains were isolated from penicillin production wastewater but they did not carry any beta-lactamase-encoding genes (Li et al. 2009). Another study reported that the prevalence of *blaTEM(1)* is greater in natural waters where anthropogenic influence is high and attributed most of penicillin resistance to the aforementioned gene (Pontes et al. 2009). From the results of PCR and phenotypic tests for beta-lactamases, it can be assumed that beta-lactamase production may not be primarily causing beta-lactam resistance in the bacterial species in this study, other resistance mechanisms, such as multidrug efflux systems, should be investigated. Although both of the ARGs were harbored by *Aeromonas,*

none of the *blaTEM* carrier strain carried the ARG *qnrS2*, hence their co-existence could not be proved. The role of *Aeromonas* as a potential reservoir and vehicle for dissemination of ARGs has been discussed and this study further confirms the environmental concerns associated with it.

Conclusion

Out of the total 48 strains tested, 29% and 31% strains exhibited the highest MIC (256 µg mL^{-1}) for fluoroquinolones and ampicillin, respectively. There were 21% strains beta-lactamase producers. Two ARGs *qnrS2* and *blaTEM* were detected in *Aeromonas* spp. and *Escherichia* sp. Although the targeted ARGs were not found to be as prevalent in the antibiotic-resistant bacteria as was expected but the role of *Aeromonas* as a vector of ARGs of clinical relevance is strengthened by the study findings. Attention should be paid, and further investigations are needed to do in aquatic environment of Pakistan to better understand the intensity of the situation. Also, the use of nonculture techniques is necessary to understand the dynamics of transfer of ARGs in the local aquatic environment.

Author contributions SS did the experimental work, MF collected the strains from wastewater, MM and RZ assisted in experimental work and preparation of the manuscript. IA, UN and MA reviewed the manuscript and improved the discussion. MA (PI) obtained research funding, supported by IA (Co-PI).

Compliance with ethical standards

Conflict of interest Authors have no conflict of interest regarding findings and publication of this research. There are no financial competing interests relevant to the work presented here.

Accession numbers The sequences of the ARGs (*qnrS2* and *blaTEM*) were deposited in Genbank under the accession numbers MK359204, MN218676 and MN218677.

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