LGU Journal of
LIFE SCIENCES

LGU Society of
Life Sciences

DOI: https://doi.org/10.54692/lgujls.2022.0604200

Research Article
LGU J. Life. Sci
Vol 6 Issue 4 October - December 2022
ISSN 2519-9404
eISSN 2521-0130

Antibiotic resistant bacteria in diverse ecological water samples are a threat to Human Food security

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ABSTRACT: *Antibiotic resistance is increasing with every passing day globally and it has become common not only in clinical settings but also in different environments. Aquatic systems are major reservoirs of antibiotic resistance genes in the environment. In this study, we have isolated the antibiotic resistance bacteria from aquatic systems including river Ravi, fish farm and underground drinking water source. Physical, chemical and microbiological parameters were studied, bacteria were isolated, gram staining and genus identification was done by following the Burgey's manual of systematic bacteriology. While, antibiotic susceptibility testing was done by following the disk diffusion method and CLSI guidelines. Multiple antibiotic resistant indexes were calculated. There was no significant discrepancy in physical and chemical parameters of water samples whereas in microbiological parameters, a variable number of bacteria were found in all 3 water samples indicating the presence of microbial contamination. A total of 33 bacterial strains were identified including Enterobacteriaceae, Pseudomonas, Bacillus, Lactobacillus and Staphylococcus species. Out of 10 classes of antibiotics, resistant against 8 classes were identified. Organisms isolated from river Ravi were the most resistant isolates. Most of the organisms were resistant to Penicillins, Carbapenems and Cephalosporins. MAR index of isolated strains ranges from 0.43 to 0.88. AMR is common in all environmental water samples indicating a threat to human food safety.*

Keyword: Antibiotic resistant bacteria (ARB), Antibiotic resistance genes (ARGs), antibiotic susceptibility testing (AST), Multiple antibiotic resistant index, Water sources

INTRODUCTION

Antibiotics are commonly used not in humans but also in animals around the globe to treat various bacterial infections. This extensive use of antibiotics has caused the emergence of resistance to almost all available antibiotics that are even considered last choice for managing certain infections. Antibiotic resistance (AR) is now common even in environmental bacteria serving as a reservoir and spreading the antibiotic resistance genes (ARGs) to pathogenic bacteria due to poor management of antibiotic use and dissemination (Mazhar et al., 2021). In recent years, the deaths caused by antibiotic resistance bacteria have reached to comparable levels to the top causes of death worldwide such as influenza, TB and HIV (Pruden et al., 2021). In United States and in Europe, the cost of treatment of patient who became ill of antibiotic resistance bacteria is reached to 2.2 billion USD and 1.5 billion EUR respectively (Lu et al., 2022). As there are multiple bacteria causing AR, so, the environmental surveillance is not possible but it is clear now that pathogenic bacteria mostly acquire ARGs from non-pathogenic environmental bacteria via mobile genetic elements thus becoming a foremost risk to animals and human wellbeing (Pruden et al., 2021). In environmental studies, main focus of researchers is typically on the presence of antibiotic genes in different environments (de Almeida Kumlien et al., 2021) and transfer of ARGs from waste water treatment plants and other food and water habitats are hotspots of environmental research currently (Calderón-Franco et al., 2021).

Antibiotic resistance bacteria are very common in different rivers across the world (Hu et al., 2021). Rivers are one of the key reservoir and drivers of ARGs in the environment and their spread to humans and animals (K. Zhang et al., 2021). River water are mostly contaminated by aquaculture waste, waste water treatment effluents and also from hospital waste (L. Zhang et al., 2021). River water in many parts of the world is used for drinking purposes and as a water supply to homes, consequently spreading the ARGs to human beings. Moreover, river water is being consumed by cattles and animals may got infection which can be transferred to humans through milk or meat (Yu et al., 2022). With the increase in population, aquacultures including fish farming have been increased tremendously to meet the food requirements. Like cattle industry, fish farmers also rely for the most part on antibiotics as prophylactic measure and increasing the growth of animals. Hence, playing a part in dissemination of ARGs not only in the environment but also to human food chain (Jo et al., 2021). Fish industry is also responsible for spreading the ARGs to drinking water sources due to the mixing and leaching of polluted water to underground and surface water reservoirs if proper measures are not taken (Liu et al., 2021; Zhou et al., 2021). Presence of many antibiotic resistance bacteria have been reported globally in drinking water supply, thus creating a challenge for human fitness internationally. Transport and fitting of water pipelines also playing a role in transferring the antibiotic resistance bacteria in drinking water systems (Hu et al., 2021).

In this study, we have checked the presence of bacteria and their antibiotic resistance determinants from river Ravi water – a large river in Punjab, Pakistan. Water of this river is being used for cleaning and bathing animals and sometimes animals drink it too in little quantities while bathing, from fish pond water in Lahore and from a hand pump water sample that is being used for drinking purposes (both animals and humans) by local people. Fish and cattle are important source of meat/food for humans' likewise drinking water. Presence of microbial contamination in these food and water sources may impose potent risk to humans. The objective of this study was to identify and report the potential presence of microbial contamination in food/water sources of humans.

MATERIAL and METHODS

Water Sampling

Using sterile bottles, water samples from 3 different locations in Lahore, Pakistan were collected. First sample was collected from a Fish farm located in Lahore, second water sample was collected from river Ravi and third sample was collected from a hand pump in Lahore to check the bacterial community in underground water.

Water Quality Parameters

Physical, chemical and microbiological parameters for water quality were tested after collection of samples from each sampling site. Samples were processed within 4 hours after sampling. Physical parameters including color, odor, temperature, electrical conductivity, pH, turbidity, and chemical parameters including alkalinity, bicarbonate, calcium, carbonate, chloride, hardness, magnesium, nitrate, potassium, sodium, sulphate, and total dissolved solids were tested respectively. In microbiological analysis presence of total coliforms, fecal coliforms and *E.coli* were tested (Henderson et al., 2022).

Physiological and morphological characterization

In order to identify and characterize bacteria from water samples, samples were further plated using nutrient agar. From fish farm water sample and hand pump water sample, direct spreading of 50µl water was carried out in triplicate on nutrient agar. Whereas water from river Ravi was visibly muddy, so, it was placed on shelf for half an hour and after settling down of mud and visible impurities, serial dilutions were made carefully (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) and then 50 µl of each dilution was plated in triplicate and incubated at 37°C for 24 hours. After 24 hours, colony forming units (CFU/ml) of each plate were counted. Colonies having different and distinct morphologies were selected and purified after noting down the colony morphologies. Next day, purified colonies were used for Gram staining and observed under light microscope (Poonia et al., 2014).

Identification of bacteria and Antibiotic Susceptibility Test

Each isolated bacteria was identified up to the genus level using the Burgey's manual. Kirby Bauer disc diffusion method was used for antibiotic susceptibility testing. McFarland 0.5 standard was used to optimize the bacterial cultures. Zones of Inhibition were measured and results were recorded according to the Clinical & laboratory standards Institute (CLSI) guidelines (Poonia et al., 2014).

Multiple Antibiotic Resistance (MAR) Index

All of the antibiotic resistant bacterial strains were analyzed for multiple antibiotic resistant index (Sinegani et al., 2017) by using the following equation:

$$MAR = \frac{\text{Number of antibiotics to which an isolate is resistant}}{\text{Total number of antibiotics exposed to bacterial strain}}$$

RESULTS

Water Quality Parameters

In physical parameters, water color was noted down after filtering the dissolved mud as it might give false coloration to water and all the samples were colorless. Fish pond water and hand pump water samples were odorless but Ravi water sample had a pungent smell. Temperature was in accordance with the climatic conditions at the time of sampling. Electrical conductivity of fish farm sample was highest while hand pump water has lowest E.C value. pH of 3 water samples ranges between 6.8 – 7.9 whereas the turbidity of fish farm water and Ravi water was quite high as compared to permissible limits (Table 1).

In chemical analysis, alkalinity, bicarbonate, calcium, chloride, hardness, magnesium, nitrate, potassium, sodium, sulphate, and total dissolved solids were all positive in different ranges except the carbonate which was absent in all 3 samples as described in Table 1. None of the chemical parameters were elevated above the permissible limits.

Table 1: Comparison of Water Quality Parameters

Parameters	**Units**	**Detection Limit**	**Reference Method**	**Permissible limits (PSQCA/ NSDWQ, 2010)**	**Permissible limits (WHO)**	**Fish Farm**	**Hand Pump**	**River Ravi**
Physical Analysis								
Color	-----	-----	Sensory Evaluation	5	5	Colorless	Colorless	Colorless
Odor	-----	-----	Sensory Evaluation	Odorless	Odorless	Pungent	Odorless	Pungent
Temperature	°F	41	Thermometer	NGVS	NGVS	61	58	63
Electrical Conductivity (E.C)	(μS/cm)	0.11	APHA, 22nd Edition	NGVS	NGVS	1076	282	434
pH	-----	0.03	APHA, 22nd Edition	6.5-8.5	6.5-8.5	7.90	6.80	7.40
Turbidity	NTU	0.31	APHA, 22nd Edition	<5	0.5	8.10	1.25	10.12

Chemical Analysis								
Alkalinity as $CaCO_3$	ppm	-----	APHA, 22nd Edition	NGVS	NGVS	8.8	2.6	04
Bicarbonate	ppm	5.0	APHA, 22nd Edition	NGVS	NGVS	440	130	200
Calcium	ppm	2.0	APHA, 22nd Edition	NGVS	200	44	32	44
Carbonate	ppm	5.0	APHA, 22nd Edition	NGVS	NGVS	Nil	Nil	Nil
Chloride	ppm	2.0	APHA, 22nd Edition	250	250	29	09	19
Hardness	ppm	5.0	APHA, 22nd Edition	500	500	260	115	170
Magnesium	ppm	1.0	APHA, 22nd Edition	NGVS	150	36	8.5	15
Potassium	ppm	0.02	APHA, 22nd Edition	NGVS	30	02	01	02
Sodium	ppm	1.57	APHA, 22nd Edition	NGVS	200	146	20	30
Sulphate	ppm	0.24	APHA, 22nd Edition	NGVS	250	130	22	20
Nitrate	ppm	0.03	APHA, 22nd Edition	10	10	2.34	0.28	0.89
Total Dissolved Solids (TDS)	ppm	-----	APHA, 22nd Edition	1000	1000	662	172	254
Microbiological Analysis								
Total coliforms	CFU/100 ml	<1.0	Colilert Quantitray – 2000 (AOAC)	0 (NSDWQ, 2010)	0/250 ml	12	68	189
Fecal Coliforms	CFU/100 ml	<1.0	Colilert Quantitray – 2000 (AOAC)	0 (NSDWQ, 2010)	0/250 ml	0	21	112

E.coli	+ve/-ve	-----	APHA, 22nd Edition FAO,1998 (International method based on APHA Standard Method: 9221B-C)	-ve (NSDWQ, 2010)	-ve	-ve	+ve	+ve

WHO: World Health Organization

APHA: American Public Health Association

AOAC: Association of Official Analytical Chemists

CFU: Colony Forming Unit

FAO: Food & Agriculture Organization

NSDWQ: National Standards for Drinking Water Quality

USEPA: United States Environmental Protection Agency

PSQCA: Pakistan Standard Quality Control Authority

NGVS: No Guideline value set

In microbiological analysis, results showed the presence of microbes in all water samples. Coliforms were present in all 3 water samples, whereas *E. coli* and fecal coliforms were only present in river Ravi sample and hand pump sample. The microbial load is very high in Ravi water whereas it is intermediate in hand pump sample and lowest in fish farm sample indicating the presence of contamination in all 3 sites but fish farm sample was free of fecal contamination.

Physiological and morphological characterization

Colony forming unit (CFU/ml) of each sample and dilution is summarized in Table 2.

Table 2: CFU Count of samples

Water Sample	Dilution	CFU in Plate 1	CFU in Plate 2	CFU in Plate 3	Average CFU
River Ravi	0	TNTC	TNTC	TNTC	TNTC
	10^{-1}	TNTC	TNTC	TNTC	TNTC
	10^{-2}	TNTC	TNTC	TNTC	TNTC
	10^{-3}	TNTC	TNTC	TNTC	TNTC
	10^{-4}	98	106	144	116
Fish Farm	0	TNTC	278	265	271.5
Hand Pump	0	69	35	58	54

TNTC: Too numerous to count

Morphological diverse strains were selected from each countable plate and purified. A total of 33 strains were selected from 3 water samples for further study. From Ravi water sample, 36.4% strains were selected whereas from fish farm and hand pump samples, 33.3% and 30.3% strains were selected respectively. Strains vary in their pigmentation ranging from white, off-white, yellow, orange and reddish brown. Among the selected isolates, 49% were gram negative rods, 39% were gram positive rods and 12% were gram positive cocci. No gram-negative cocci were observed.

Bacterial genus identification

In order to differentiate between gram positive rods, spore staining was done. Spore staining was positive in 31% isolates whereas 69% isolates were negative for spore staining. Spore formers were further checked for the growth in anaerobic conditions but none of the isolate was grown in anaerobic conditions confirming their genus to be *Bacillus*. Non spore formers were checked for acid fastness to rule out the *Mycobacterium* species. None of the isolate was positive for acid fast staining. These strains were further checked to determine the presence of catalase. 22% isolates were positive for catalase and 78% were negative. For gram positive cocci, catalase test was performed confirming all of the isolates to be catalase producers. Mannitol fermentation was carried out which is positive in 50% of isolates confirming them to be *Staphylococcus aureus*. Furthermore, yellow pigment was observed to rule out the Micrococcus species, none of the isolate was positive for yellow pigment hence confirming the presence of other *Staphylococcus* species.

For gram negative rods, oxidase test was performed, 46% isolates were positive for the said enzyme. Glucose fermentation was tested and 67% isolates were negative confirming them to be *Pseudomonas* species. Glucose fermenters were analyzed for their growth requirements of Na^+ and none of them required sodium for their growth confirming them to be *Aeromonas* species. For oxidase negative, gram negative rods of family *Enterobacteriaceae,* API 20E strips (Biomerieux) were used which showed the presence of genus *Escherichia, Proteus, Serratia, Klebsiella, Citrobacter and Enterobacter*.

Antibiotic susceptibility Testing

A total 10 classes of antibiotics were checked by following the Clinical and Laboratory Standards Institute’s guideline 2019. Out of 10 classes, resistance to 8 classes was determined (Table 3). Out of 7 major classes of antibiotics, resistance against 6 classes was present. Resistance to penicillins was highest in all isolates even 4th generation penicillin resistant was observed frequently. Resistance to Cephalosporins and Carbapenems was also very high among all isolates. Resistance to Quinolones was also observed in large number of isolates. Tetracycline and Aminoglycosides resistance is less common as compared to other antibiotic classes. No resistance to Chloramphenicol was observed. Other antibiotics such as Macrolides, Oxazolidinones also showed resistance greatly whereas no resistance to Lincosamide was observed (Table 3).

Table 3: Antibiotic Susceptibility Test Results

Antibiotic Class	Antibiotic Generation	Antibiotic (Conc. In μg)	Abbreviation	Total No. of isolates tested	Percentage of Isolates (No. Of Isolates)		
					Resistant	Intermediate	Sensitive
Penicillins	2nd	Amoxicillin (2)	A2	16	100 (16)	0	0
	2nd	Amoxicillin + Clavulanic acid (2+1)	AUG3	11	100 (11)	0	0
	3rd	Ampicillin (10)	AM10	11	73 (8)	0	27 (3)
	4th	Piperacillin 30	PRL30	13	100 (13)	0	0
	4th	Piperacillin-Tazobactam (30+6)	PTZ36	5	100 (5)	0	0
Aminoglycosides	-----	Gentamycin (10)	GM10	11	36 (4)	9 (1)	55 (6)
	-----	Tobramycin (10)	TN10	4	25 (1)	50 (2)	25 (1)
	-----	Amikacin (30)	AK 30	10	40 (4)	20 (2)	40 (4)
Tetracycline	1st	Tetracycline (30)	TE30	13	38.5 (5)	23 (3)	38.5 (5)
	2nd	Doxycycline (30)	DX30	2	0	0	100 (2)
Quinolones	2nd	Ciprofloxacin (1)	CIP1	15	73 (11)	20 (3)	7 (1)
	2nd	Levofloxacin (10)	LEV10	19	37 (7)	37 (7)	26 (5)
	3rd	Gatifloxacin (5)	GAT5	13	85 (11)	15 (2)	0
Cephalosporins	2nd	Cefuroxime (5)	CXM5	15	100 (15)	0	0
	2nd	Cefoxitin (10)	FOX10	16	100 (16)	0	0
	3rd	Cefotaxime + Clavulanic Acid (30+10)	CTC40	22	55 (12)	0	45 (10)
Macrolides	-----	Azithromycin (15)	ATH15	13	61 (8)	8 (1)	31 (4)

Carbapenems	-----	Meropenem (10)	MEM10	13	100 (13)	0	0
	-----	Imipenem (10)	IMI10	8	87.5 (7)	12.5 (1)	0
Lincosamide	-----	Clindamycin (2)	CD2	3	0	67 (2)	33 (1)
Chloramphenicol	-----	Chloramphenicol (30)	C30	13	0	46 (6)	54 (7)
Oxazolidinones	-----	Linezolid (10)	LZD10	2	100 (2)	0	0

All of the isolated strains were resistant to multiple antibiotics confirming the presence of large number multi-drug resistant isolates. None of the strain was resistant or sensitive to all antibiotics.

Multiple Antibiotic Resistance (MAR) Index

In all 33 strains, none of the bacterial isolate has an MAR index of 1. In hand Pump water sample, MAR ranges from 0.5 to 0.75, in fish farm water MAR ranges from 0.5 to 0.88 and in river Ravi water sample MAR ranges from 0.43 to 0.88 (Fig 1).

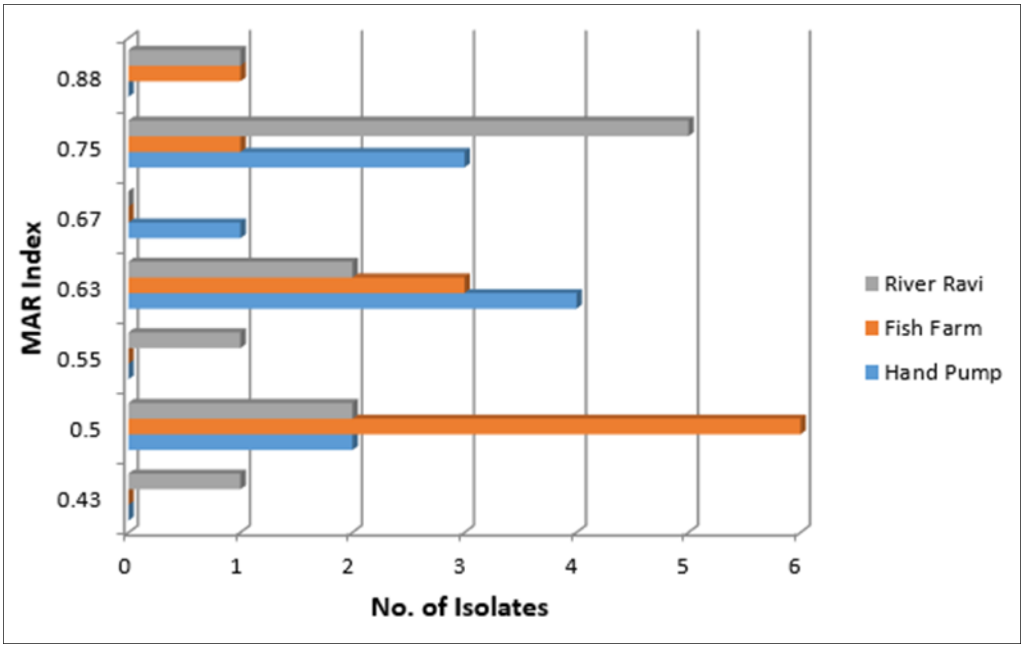

Fig 1: MAR Index of bacteria isolated from different water sources

DISCUSSION

Physical parameters of water quality were checked of all the 3 samples. All the samples were colorless indicating the absence of any colored salts or impurities. Ravi water had pungent smell which may attributed to the presence and decay of organic products whereas fish farm sample and hand pump sample (bore water) were odorless (absence of organic matter and decaying bacteria). Temperature of all samples was in accordance with the atmospheric temperature. pH is an indicator of corrosive nature of water and can be toxic to fish. Higher pH is due to high carbon dioxide and low oxygen dissolution (Patil et al., 2012). pH of all our samples was also in acceptable limits. Electrical conductivity is an indicator of the presence of ions in a water sample. Fish farms have highest E.C value indicating the presence of high ionic species in it which is indirectly an indicator of contamination (Mahajan et al., 2006). Turbidity is an indicator of dissolved mud or other impurities in water. Higher turbidity means more presence of impurities or mud.

Chemical parameters of all the samples were in permissible limits. Alkalinity is a measure of stability of pH of water. If pH of samples is above 8.3, it is the indication of presence of carbonates, pH below 8.3 is indicator of the presence of bicarbonates (Patil et al., 2012). Our sample's pH ranges between 6.8-7.9 and indicating the absence of any carbonates. All other chemical parameters were also in permissible limits. Our main focus was on the biological contamination in water sources as we have studied diverse water samples not only drinking water samples. Physical and chemical parameters should be in normal range as of fish farm water, survival of fish is dependable on these parameters whereas Ravi water is being consumed by cattles and for irrigation purposes.

In bacteriological parameters, coliforms were identified in all three samples whereas fecal coliforms were identified in 2 out of 3 samples. Presence of *E.coli* and fecal coliforms were confirmed in river Ravi water and hand pump water. Contamination of microbes and fecal coliforms in hand pump water sample is an indication of seepage of contaminated Ravi water in underground drinking water source which is quite alarming in terms of water purity and safety.

Environmental sources like soil and water reservoirs are the major sites where horizontal gene transfer of different genes takes place, including antibiotic resistance gene transfer. Number of ARBs related morbidities and mortalities are increasing day by day and it was estimated that mortalities due to ARBs will become the leading cause of death by 2050. WHO has made a plan to control the ARB related mortalities and morbidities by minimizing the use of antibiotics and an increased surveillance of clinical as well as environmental antibiotic resistance bacteria (Henderson et al., 2022). Many bacteria resistant to antibiotics and multidrug resistant bacteria were identified in this study. This showed the high prevalence of drug resistant bacteria in environmental water sources. Thirty-three bacterial isolates were chosen randomly and these bacteria were identified to be the members of 12 different genera. In our study, we have found out the most frequent genus were *Bacillus, Escherichia, Pseudomonas and Staphylococcus*. A study from Ethiopia has reported the frequent genera to be *Providencia, Kluyvera, Citrobacter, Escherichia and Aeromonas* (Gessew et al., 2022). *Escherichia coli* and *Klebsiella* species were found in greater numbers in many other studies reported from India, Bangladesh and Ethiopia and a study from Ethiopia also reported the prevalence of *Salmonella* species along with the above mentioned species (Tesfaye et al., 2019). Another study from Poland has reported the presence of *Escherichia, Citrobacter, Klebsiella, Enterobacter, Kluyvera, and Raoultella* species which was relatively closer to diversity of our isolates (Korzeniewska et al., 2013).

All isolates were tested against multiple antibiotics depending upon their genus and CLSI guidelines. Maximum number of antibiotics used against a single isolate was 11 and minimum 3 antibiotics were checked against a single isolate. Resistance against Amoxicillin (2µg), Amoxicillin-clavulanate (2+1 µg), Piperacillin (30µg), Piperacillin-Tazobactam (30+6 µg), Cefuroxime (5 µg), Cefoxitin (10 µg) and Meropenem (10 µg) were 100%.

MAR Index is an indicator of the risk of pollution or contamination in water due to frequent use of antibiotics or other anthropogenic dealings (Amador et al., 2015). MAR index value of above 0.2 is an indication of exposure of high-risk sources of animal and human contamination wherever exploitation of antimicrobials is regular and common. On the contrary, MAR index of below and equals to 0. 2 is an indication of rare use of antibiotics or complete absence of antibiotics in a sample (Gessew et al., 2022). In general, MAR index of 0.4 and above is an indicator of human fecal contamination whereas MAR index below 0.4 is an indicator of fecal contamination from non-human sources (Gessew et al., 2022; Poonia et al., 2014). In our study, all of the isolates have a MAR index of above 0.4 which is quite alarming and precautionary measures should be taken.

CONCLUSION

Enterobacteriaceae, Pseudomonas, Bacillus, Lactobacillus and Staphylococcus species were identified in all the water samples altogether. Resistance against antibiotics was found in all the organisms isolated from all 3 water samples. Highest resistance was found in river Ravi isolates. Resistance against Penicillins was most common in isolated strains followed by carbapenems and cephalosporins. MAR Index of all the isolates was higher than 0.4 indicating a threat to human food security.

ACKNOWLEDGEMENTS

This manuscript is the part of the Ph.D. thesis of author Hafiza Jawairia Hashmi. Authors are obliged to the University of the Punjab Lahore Pakistan for financial support.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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