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ISOLATION AND CHARACTERIZATION OF MULTI-DRUG RESISTANT (MDR) AND EXTENSIVELY DRUG RESISTANT (XDR) BACTERIA FROM DIVERSE ENVIRONMENTAL NICHES

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Keywords:

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ABSTRACT: Antimicrobial resistance (AMR) emerged as a major challenge to public health and significantly impacting the global economy. Infections due to multi-drug resistant (MDR) bacteria are difficult to treat and cause financial burden on patients. Environment may serve as a genetic pool of several drug resistant genes and help bacteria to emerge as notorious superbugs. This study aimed to characterize MDR bacterial isolates from environmental samples and comparative analysis of MDR and multi-drug sensitive (MDS) strains for the expression of virulence factors. Twenty six (n=26) different environmental samples (water, soil, air and surface) were collected for the purpose. The antimicrobial susceptibility testing performed using 12 antibiotics belongs to six different antibiotic classes. The potential virulence factors were determined among MDR and MDS isolates. Thirty (n=30) bacterial isolates belong to 13 different genera (*Staphylococcus spp., Enterococcus spp., Escherichia spp., Bacillus spp., Acinetobacter spp., Klebsiella spp., Pseudomonas spp., Shigella spp., Enterobacter spp., Citrobacter spp., Streptococcus spp., Streptobacillus spp.* and *Proteus spp.*) were identified. All recovered isolates were susceptible to aminoglycosides. The most frequently (23.33%) isolated bacterium was *Staphylococcus aureus*. Overall, 63.33% (19/30) bacterial isolates were MDR, of which, 73.68% (14/19) were extensively drug resistant (XDR). Biofilm production observed in all the isolates as weak (53.34%) and moderate (46.66%). MDR plus XDR phenotype was observed among 42.85% (6/14) of the moderate biofilm producers. Statistically, no significant difference was observed between MDR and MDS isolates for the expression of virulence traits ($p>0.05$ for calculated χ^2 of 8.496). Incidence of MDR and XDR phenotype was higher among isolates recovered from water samples and was least among surface samples. Release of such notorious superbugs in the environment should not be overlooked and biomedical waste management (BMW) rules must be followed.

INTRODUCTION: Antibiotic is among the most revolutionary medical discoveries. Alexander Fleming introduced first widely used antibiotic, penicillin in 1928.

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But later, in 1950s, it was discovered that *Staphylococcus aureus* was producing penicillinase, an enzyme that acts on β-lactam ring of antibiotic and renders the antibiotic ineffective.

This led to the emergence of multidrug resistant *S. aureus*. The primary forces behind the evolution of drug resistance are antimicrobial selection pressure and the proliferation of resistant organisms. There are mainly four most frequent drug resistance mechanisms *i.e.* limiting drug uptake, altering the drug target, activating drug efflux, and inactivating

drug. These methods could either be innate or acquired from other microbes through horizontal gene transfer-mediated acquisition of extra-chromosomal genetic components [1]. The major contributing factors for antimicrobial resistance are indiscriminate use of antibiotics, their accessibility over the counter and their usage as growth boosters in agricultural and animal husbandry [2, 3]. Moreover, non-selective nature of broad-spectrum antibiotics is also contributing towards development of drug resistance as it kills our normal flora as well, in addition to the pathogen that is causing the ailment. Alternative methods need to be explored to combat drug resistant pathogens as there will be no effective antibiotic available if no new drug is developed or discovered by 2050 [4].

Drug resistant microbes cause infections which are difficult to treat and cause prolonged hospital stay which ultimately increase the financial burden. Antibiotic resistance occurs through genetic modifications. Antibiotic resistance genes (ARG) are transmissible among bacteria and are mediated by the horizontal gene transfer [5]. Besides this, new resistance mechanisms being continuously evolved in bacteria, this might impose a danger to our ability to fight against prevalent infectious diseases [6].

Antibiotics can be categorized by several ways but the most common classification schemes are based on their molecular structures, mode of action and spectrum of activity. Some most common classes of antibiotics based on chemical or molecular structures include Beta-lactams, Macrolides, Tetracyclines, Quinolones, Aminoglycosides, Sulphonamides, Glycopeptides and Oxazolidinones [7]. European Centre for Disease Control (ECDC) and Centre for Disease Control & Prevention (CDC), Atlanta, defined multidrug resistance (MDR) as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. Both the organizations described extensively drug resistance (XDR) as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (*i.e.*, bacterial isolates remain susceptible to only one or two antimicrobial categories) and pan-drug resistance (PDR) as non-susceptibility to all agents in all antimicrobial categories [8]. Multidrug-resistant (MDR) bacterial infections are more prevalent these days and pose a significant threat to public health [4]. Initially, MDR microorganisms were only found in clinical isolates, but nowadays, drug resistant strains can be found in every ecological niche due to horizontal gene transfer. Environment serves up as a genetic pool of antibiotic resistant genes (ARGs) whereby human pathogens can acquire resistance genes from environmental bacteria and become superbugs. Most of the genes responsible for antimicrobial resistance (AMR) evolves in the natural environment due to the presence of naturally occurring antibiotics (including penicillin, streptomycin, tetracycline and chloramphenicol) derived from soil-dwelling bacteria and fungus as a means to compete with other bacteria for limited resources [9].

ARGs conferring resistance to a wide variety of antibiotics have been identified in wide range of environments including drinking water in both developed and developing countries [10]. According to an environmental study, MDR *Shigella boydii* and *Vibrio cholerae,* were found in samples of sewage and drinking water, suggesting the transfer of mobile resistance components between species [11].

The two most prevalent bacterial infections are vancomycin-resistant *Enterococcus* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) and they have also been isolated from environment samples such as water, animals and foods of animal origins [4]. Antimicrobial resistance and infection control are interconnected concerns that can only be properly addressed through a health system's approach, as evidenced by the emergence of multi-drug resistance and its global expansion [2]. For any infection control program, surveillance is a critical and important component to detect MDR pathogens, monitor epidemiologic trends and measure the effectiveness of interventions [12]. All stakeholders involved in human and animal health must work together in harmony at the local, national, and international levels to guarantee that patients are given the proper care they need right now and to preserve the life-saving potential of antibiotics for future generations [2]. Although there are several studies assessing multidrug resistance (MDR) in microbial populations of animal origin, not much work has been done so far on the occurrence of

MDR in different ecosystems. The present study evaluated the antibiotic resistance among aerobic mesophilic bacteria recovered from environmental sources and their association with virulence factors. This would help in understanding the role of virulence factors in the disease severity caused by MDR isolates and further management of treatment outcomes.

MATERIALS AND METHODS:

Study Site and Sampling: Twenty six (n=26) different environmental samples including water (6), soil (9), surface (10) and air (1) were randomly collected from different locations of Shimla (Himachal Pradesh) and Amritsar (Punjab) during June-July 2022. Water and soil samples were collected in sterilized containers and sampling from inanimate surfaces was done using sterilized swabs dipped in physiological saline solution. Air sampling was done from the microbiology research lab and the surface samples were also collected from premises of the Department of Microbiology, Himachal Pradesh University (Shimla).

Sample Processing, Isolation and Identification of Bacteria: Samples collected from local areas were transported to the Microbiology laboratory, Himachal Pradesh University in cool conditions and processed within two hours of collection. Outside samples were shipped to the laboratory in cold chain within 3 days of collection. Soil and water samples were serially diluted in 10-fold physiological saline and 1.0 mL aliquots of appropriate dilutions (10^{-2}–10^{-6}) were inoculated in nutrient broth tubes for enrichment purpose and incubated at 37° C for overnight. A volume of 0.1 mL of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions were plated out on nutrient agar plates and incubated under aerobic condition at 37°C for up to 48 h for maximum recovery of aerobic mesophilic bacteria. Different colonies were further purified by streaking them on nutrient agar plates.

Based on the colony morphology, representative colonies were picked and further streaked on nutrient agar plates to get purified colonies. Morphological characteristics were evaluated by microscopic analysis of Gram's-stained preparations and isolated organisms were further identified biochemically in a systematic way following standard procedures [13]. The findings were interpreted as per Bergey's Manual of Determinative Bacteriology, Volume 9 [14]. The purified colonies were stored in 10% glycerol stocks and kept at -20°C for further use.

Antimicrobial Susceptibility Testing: The standard Kirby-Bauer disc diffusion method was used to determine the antimicrobial susceptibility profiles of the isolates [15]. Bacterial inoculum was prepared by suspending the freshly grown bacteria in 4–5 ml sterile nutrient broth and the turbidity was adjusted to that of a 0.5 McFarland standard.

The antimicrobial susceptibility testing was performed on Mueller-Hinton agar plates using HiMedia dodecaUniversal-VII antibiotic discs (DE015-1PK) impregnated with broad spectrum antibiotics belongs to six different antibiotic classes namely aminoglycosides, fluoroquinolones, sulfonamides, macrolides, tetracyclines and synthetic oxazolidinones. The concentrations of 12 different antibiotics used were: Gentamicin (10µg), Netillin (30µg), Nalidixic acid (30µg), Kanamycin (30µg), Amikacin (30µg), Co-Trimoxazole (25µg), Tobramycin (10µg), Clarithromycin (15µg), Nitrofurantoin (300µg), Streptomycin (10µg), Oxytetracycline (30µg) and Furazolidone (50µg). The plates were incubated aerobically at 37°C for 18–24 hours. The zones of inhibition were measured and compared with standard guidelines [16].

Determination of Virulence Factors: The presence of potential virulence factors among environmental isolates were studied following standard methodology [17].

Gelatinase Production: The gelatinase test medium was prepared by adding 2% bacteriological gelatin (Loba Chemie, Mumbai) to nutrient agar medium (HiMedia, Mumbai). Bacterial isolates were streaked on the medium and incubated for 24 h to 72 h at 37 °C. Zone of clearance around the line of growth indicated gelatinase production.

Protease Production: The protease test medium was prepared by adding 2% skimmed milk to nutrient agar (Himedia, Mumbai). Inoculum of the test organisms in a 30µl volume was loaded in each well and incubated for 24 h to 72 h at 37 °C.

Clear zone around bacterial colonies indicated protease production.

Lipase Production: Egg yolk agar medium was prepared by mixing a sterile egg yolk (1:1) in physiological saline solution. 10% egg yolk was mixed with autoclaved nutrient agar enriched with 1% NaCl.

The contents were poured into petri plates and inoculum (30 µl) of the test organisms was placed in the wells and incubated at 37 °C for 1-4 days. Clear zone around the colonies indicated production of lipases by the organism.

Biofilm Production: Inoculum was prepared by adding loopful of each test organism in 10 ml of trypticase soy broth (TSB) tubes containing 1% glucose + 2% sucrose. Using micropipette each inoculum (200µl) was transferred to 96 well microtitre plate and incubated it at 37 ºC for 24 h. The contents were then decanted off and the biofilm washed in phosphate buffer saline (pH 7.3), dried and stained with crystal violet (0.1%). Excess of the stain was washed in deionized water. Optical density (OD) of the stained adherent bacteria was determined with a microplate reader at a wavelength of 590nm.

The results were recorded after subtracting the reading of negative control (TSB+1% glucose+2%sucrose without bacterial cells). A three-grade scale was used to evaluate the biofilm producing ability of the test organisms as follows; no or weak: OD<0.120; moderate: 0.120<OD<0.240; strong: OD>0.240 as stated by Christensen *et al* 1985 [18].

Statistical Analysis: Chi square (χ^2) test was applied to determine the significant relation between the resistant (MDR and XDR) phenotype and the expression of virulence factors. The observed values used for computing chi square at degree of freedom 12 and level of significance (p-value) 0.05 are given in **Table 1.**

TABLE 1: TABLE OF OBSERVED VALUES USED FOR CALCULATING CHI SQUARE (X^2) VALUE WITH 12 DEGREE OF FREEDOM AT 0.05 LEVEL OF SIGNIFICANCE

Bacterial phenotype	Weak Biofilm (WB)	Moderate Biofilm (MB)	Protease (P)	Lipase (L)	Gelatinase (G)	WB+P+L	MB+P+L	Total
MDS	1	4	2	2	0	2	0	11
MDR	2	1	0	2	0	0	0	5
MDR+XDR	2	1	4	3	0	4	0	14
Total	5	6	6	7	0	6	0	30

RESULTS:

Confirmation of the Environmental Isolates: Of the 26 different samples collected, all (100%) samples were positive for one or more isolates. Among the total samples, a total of thirty (n=30) mesophilic bacterial isolates were recovered including 6 (20%) from water, 11 (36.66%) from soil, 12 (40%) from surface and 1 (3.33%) from air.

On the basis of microscopic and biochemical characterization, 13 different genera were identified which include *Staphylococcus spp., Enterococcus spp., Escherichia spp., Bacillus spp., Acinetobacter spp., Klebsiella spp., Pseudomonas spp., Shigella spp., Enterobacter spp., Citrobacter spp., Streptococcus spp., Streptobacillus spp.* and *Proteus spp*. Of the 13 identified genera, majority (9/13) were gram negative rods, followed by gram positive cocci (3/13) and gram positive bacilli (1/13). Overall percentage of gram negative rods, gram positive rods and gram positive cocci among total bacterial isolates were 50%, 10% and 40% respectively. The details of microorganisms identified and their environmental source is presented in **Table 2.**

The most frequently isolated bacterium was *Staphylococcus aureus* (23.33%) and recovered from all the sources except water, followed by *Enterococcus spp.* (13.33%), *Escherichia coli* (10%), *Bacillus spp*. (10%), *Acinetobacter spp*., *Klebsiella spp*., *Pseudomonas spp*. (6.66% each).

Two *Shigella spp.* (6.66%) and one each of *Proteus spp*. (3.33%) and *Streptococcus spp*. (3.33%) were recovered from soil samples only. Surface sampling was the sole source for recovery of *Enterobacter*, *Citrobacter* and *Streptobacillus* species.

TABLE 2: DETAILS OF IDENTIFIED BACTERIAL GENERA, THEIR ENVIRONMENTAL SOURCE AND RATIO OF MULTIPLE DRUG RESISTANT ISOLATES

Bacterial Genera (n=13)	Environmental Source	Total Number of isolates recovered	Overall		Among MDRs	
			MDS (%)	MDR (%)	MDR+XDR (%)	MDR only (%)
Acinetobacter spp.	Water, Soil	2	0	2 (100)	1 (50)	1 (50)
Klebsiella spp.	Water, Surface	2	1 (50)	1 (50)	1 (100)	0
Escherichia coli	Water, Surface	3	2 (66.66)	1 (33.33)	0	1 (100)
Bacillus spp.	Water, Soil	3	1 (33.33)	2 (66.66)	1 (50)	1 (50)
Enterococcus spp.	Water, Soil, Surface	4	2 (50)	2 (50)	1 (50)	1 (50)
Shigella spp.	Soil	2	0	2 (100)	2 (100)	0
Staphylococcus aureus	Soil, Surface, Air	7	2 (28.57)	5 (71.42)	5 (100)	0
Streptococcus spp.	Soil	1	1 (100)	0	0	0
Pseudomonas spp.	Soil, Surface	2	1 (50)	1 (50)	1 (100)	0
Proteus spp.	Soil	1	0	1 (100)	1 (100)	0
Enterobacter spp.	Surface	1	1 (100)	0	0	0
Citrobacter spp.	Surface	1	0	1 (100)	1 (100)	0
Streptobacillus spp.	Surface	1	0	1 (100)	0	1 (100)
Total		30	11 (36.66)	19 (63.33)	14 (73.68)	5 (26.31)

Out of overall 19 multidrug resistant (MDR) isolates, 14 isolates were MDR with extensively drug resistant (MDR+XDR) phenotype and remaining 5 isolates were only MDRs without XDR phenotype.

Antibiotic Cultural Sensitivity Test: The bacterial isolates were screened for their susceptibility to different antibiotic groups that are commonly used to treat infections caused by them, by *in-vitro* culture sensitivity assay. All of the isolates of *S. aureus* were found to be resistant to nitrofurantoin followed by nalidixic acid (85.7%), co-trimoxazole, oxytetracycline, furazolidone and clarithromycin (71.4% each). Out of seven, five (71.4%) isolates of *S. aureus* were resistant to six antibiotics tested. None of the *S. aureus* isolates were resistant to gentamicin, netilin, kanamycin, amikacin, tobramycin and streptomycin. Majority (75%) of the *Enterococcus spp*. isolates were resistant to nitrofurantoin and co-trimoxazole and 50% (2/4) were multidrug resistant. Of the total 30 bacterial isolates recovered, resistance to aminoglycoside class of drug (netilin) was only observed in one isolate of *Enterococcus spp*. All bacterial isolates recovered were found to be susceptible to aminoglycoside class of antibiotics tested. Among all gram negative bacilli recovered, one isolate each of *Shigella, Pseudomonas, Proteus, Citrobacter* and *Acinetobacter* species were resistant to all antibiotic classes tested except aminoglycosides **Table 3.** Overall 63.33% (19/30) bacterial isolates were found to be multidrug resistant (MDR) and rest (11/30) were multidrug sensitive (MDS). Of the overall MDRs, 73.68% (14/19) were categorized as extensive drug resistant (XDR) and 26.32% (5/19) were only MDR phenotype **Table 2**. The distribution of MDR, MDS and XDR among gram-positive rods, gram-negative rods and gram-positive cocci is presented in **Fig. 1.** MDR and XDR percentage was higher among isolates recovered from water samples and it was recorded least among surface samples. In soil samples, the ratio of MDR and XDR isolates were 18.18% and 54.55% respectively. The distribution of MDR and XDR isolates among different sample sources is represented in **Fig. 2.**

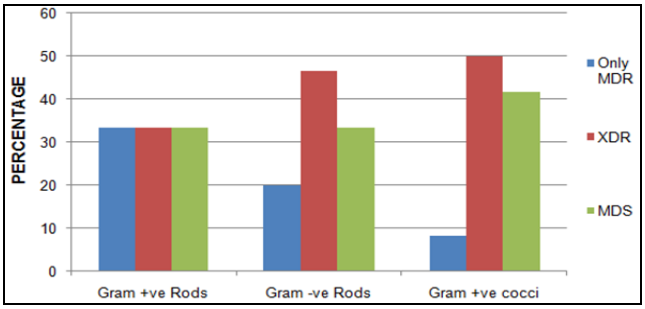

FIG. 1: DISTRIBUTION OF MDR AND XDR PHENOTYPES AMONG BACTERIAL ISOLATES

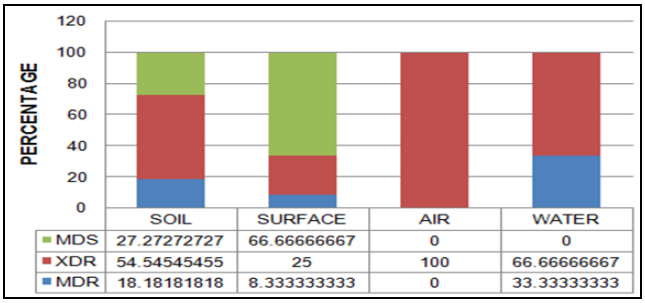

FIG. 2: MDR AND XDR RATIO AMONG DIFFERENT SAMPLE SOURCES

TABLE 3: ANTIMICROBIAL RESISTANCE PATTERN OF THE BACTERIAL ISOLATES TO DIFFERENT ANTIBIOTIC CLASSES

Bacterial isolates (n=30)	Antibiotic Classes												MRI
	AMG						MAC		FQL	SF	TC	SOX	
	GEN	K	AK	NET (%)	TOB	S	CLR (%)	NIT (%)	NA (%)	COT (%)	O (%)	FR (%)	
Staphylococcus aureus (n=7)	0	0	0	0	0	0	5 (71.4)	7 (100)	6 (85.7)	5 (71.4)	5 (71.4)	5 (71.4)	50
Enterococcus spp. (n=4)	0	0	0	1 (25)	0	0	2 (50)	3 (75)	2 (50)	3 (75)	1 (25)	2 (50)	58.33
Escherichia coli (n=3)	0	0	0	0	0	0	2 (66.6)	2 (66.6)	0	0	1 (33.3)	2 (66.6)	33.33
Bacillus spp. (n=3)	0	0	0	0	0	0	0	2 (66.6)	1 (50)	2 (66.6)	3 (100)	3 (100)	41.66
Acinetobacter spp. (n=2)	0	0	0	0	0	0	2 (100)	2 (100)	1 (50)	1 (50)	2 (100)	2 (100)	50
Klebsiella spp. (n=2)	0	0	0	0	0	0	0	1 (50)	0	1 (50)	1 (50)	2 (100)	33.33
Shigella spp. (n=2)	0	0	0	0	0	0	2 (100)	2 (100)	2 (100)	1 (50)	2 (100)	2 (100)	50
Pseudomonas spp. (n=2)	0	0	0	0	0	0	2 (100)	1 (50)	1 (50)	1 (50)	2 (100)	1 (50)	50
Proteus spp. (n=1)	0	0	0	0	0	0	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	50
Enterobacter spp. (n=1)	0	0	0	0	0	0	0	1 (100)	0	0	1 (100)	0	16.66
Citrobacter spp. (n=1)	0	0	0	0	0	0	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	50
Streptococcus spp. (n=1)	0	0	0	0	0	0	0	0	1 (100)	0	0	0	8.33
Streptobacillus spp. (n=1)	0	0	0	0	0	0	0	1 (100)	1 (100)	1 (100)	0	0	25

Different antibiotic classes namely AMG=aminoglycosides, MAC=macrolides, FQL=fluoroquinolones, SF=sulphonamides, TC=tetracyclines, SOX=synthetic oxazolidonones. [1] MRI = multiple antibiotic resistance index. [1] GEN=Gentamicin, NET=Netilin, NA=Nalidixic acid, K=Kanamycin, AK=Amikacin, COT=Co-trimoxazole, TOB=Tobramycin, CLR=Clarithromycin, NIT=Nitrofurantoin, S=Streptomycin, O=Oxytetracycline, FR=Furazolidone

Determination of Virulence Factors: Overall 40% isolates were found to express protease activity and 43.33% isolates possess lipase activity. However, gelatinase production was not observed in any of the environmental isolates. Biofilm formation was observed in all the 30 bacterial isolates examined as moderate (46.66%) and weak (53.33%) by tissue culture plate method. Of the moderate biofilm producers, 42.85% (6/14) were found to have MDR plus XDR phenotypes. Only 4/30 isolates which were protease and lipase producers with MDR plus XDR phenotype, were weak producers of biofilm. Statistically, the probability value (p-value) for a χ^2 of 8.496 with 12 degree of freedom corresponds to a probability of more than 0.05 but less than 0.75. Hence, resistant phenotype had no significant relation with the expression of virulence factors, as p>0.05 for calculated χ^2 of 8.496 **Table 4.**

TABLE 4: RELATION BETWEEN RESISTANT PHENOTYPE AND VIRULENCE FACTORS EXPRESSION AT DIFFERENT SIGNIFICANCE LEVELS

Significance level (p value)	χ^2 tabular (degree of freedom 12)	Significant relation between both variables
0.05	21.03	No
0.75	8.438	Yes
0.90	6.304	Yes
Calculated χ^2 value is 8.496 p>0.05, degree of freedom=12, Null hypothesis accepted		

DISCUSSION: The present study aimed to determine the presence of multidrug resistant phenotype among the aerobic mesophilic bacteria recovered from environmental sources and their

susceptibility patterns to different antibiotics classes. In this study, 13 different bacterial genera were identified from 26 different environmental samples collected and a total of 30 bacterial isolates were recovered. Gram-negative rods were found in majority (15/30) of the isolates and among them 50% were recovered from surface sampling. A similar trend was reported that 86% of the isolated Gram-negative bacilli were recovered from environmental surfaces in the intensive care units of hospitals [19].

The gram-negative rods recovered from surface sampling were found to be multidrug sensitive (MDS) except one isolate of *Citrobacter spp.* which was recovered from surface sampling of departmental washbasin. In contrast the gram-negative isolates from water and soil were multidrug resistant (MDR). This difference may arise due to single site selected for surface sampling *i.e.* departmental premises only while water and soil samples were collected from multiple sites including sewage drainages, pharmaceutical waste sites *etc*.

Drug resistant strains might be less probable in sampling from single source as compared to multiple sites sampling. Most frequently isolated bacterium was *Staphylococcus aureus* (7/30) and it was found that 71.42% (5/7) among them were XDR. As air sampling and surface sampling was done from the departmental premises only and the presence of XDR strains on the classroom desk and door handles may serve as the potential source of spreading the infection due to these strains.

The common sources of XDR strains were found to be on the classroom benches, door handles, sink and dustbins. The department needs to strengthen an appropriate cleaning activity, maintaining hand hygiene and periodic fumigation of lab area to contain the spread of these strains in the community. Some studies on the line also supportive of the evidence that *Staphylococcus spp.* were the most common lab contaminants [20-22].

Our study revealed, overall 19/30 (63.33%) isolates as multidrug resistant (MDR) and among them 14/19 (73.68%) as extensively drug resistant (XDR) isolates. Moges *et al* (2014) from Northwest Ethiopia reported the higher (81.5%) percentage of MDR in the waste water samples from hospital environment and lower (54.2%) percentage from non-hospital environment [23]. The contamination of waste water by antibiotics or other pollutants lead to the rise of resistance due to selection pressure. Our data also revealed that the percentage of MDR and XDR phenotypes was higher among water samples which are comparable to the findings reported by other studies from India [8, 24-25] and other countries [26-28].

The presence of antibiotic resistance organisms in water should not be overlooked and proper management of waste water and improved sanitary measure should be practiced. The antimicrobial susceptibility profiles revealed that the environmental isolates were most sensitive to aminoglycoside class of antibiotics namely gentamicin, netilin, kanamycin, amikacin, tobramycin and streptomycin, as none of the environmental isolate recovered were found to be resistant to this class of antibiotics except one isolate of *Enterococcus spp.* **Table 3** & **Fig. 3.**

Several studies from the United States and Europe have also reported high susceptibility rates for aminoglycosides (amikacin and gentamicin) against key gram-negative pathogens as per Clinical and Laboratory Standards Institute (CLSI) criteria [29].

Amikacin was one of the few agents that showed good antimicrobial activity against *P. aeruginosa* isolates from the United States. Similarly, higher aminoglycoside susceptibility rates were found among isolates collected from U.S. medical centers [30].

In contrast to this, one study from North-eastern India reported high level aminoglycoside resistance (79.2%) with coexisting extended-spectrum beta-lactamases and metallo-beta-lactamases producing strains of *Acinetobacter baumannii* from ICU Patients [31].

Mir *et al* (2016) from Chennai (India) also reported a resistance level of 72.15% for streptomycin, 73.4% for gentamicin, 63.26% for neomycin, 57.14% for tobramycin, 47.9% for netilmicin and 8.16% for amikacin in *E. coli* from urinary tract infections (UTI) patients [32].

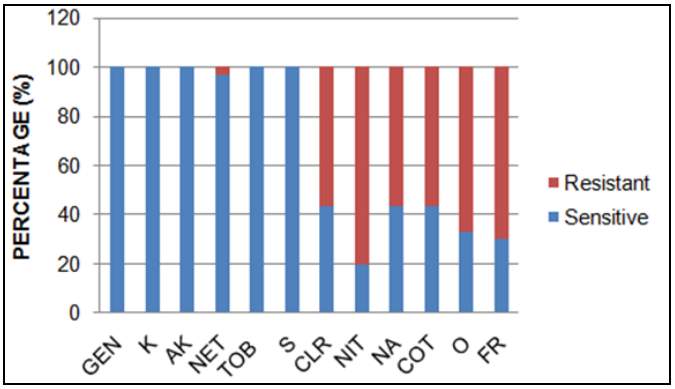

FIG. 3: ANTIMICROBIAL SUSCEPTIBILITY PROFILE: ALMOST 100% OF ENVIRONMENTAL ISOLATES WERE SENSITIVE TO AMINOGLYCOSIDES (GEN, K, AK, NET, TOB, S) CLASS OF ANTIBIOTICS

This discordance with our study reflects the low level use of aminoglycosides in the study area for treating bacterial infections or there may be a difference in antimicrobial resistance pattern among clinical and environmental isolates. Yang *et al* (2009) from Taiwan have also observed a significant difference for antimicrobial resistance rates between clinical and sewage isolates from the same hospital [33].

Although, there is limited information available on antimicrobial resistance (AMR) pattern among environmental isolates from India as only few studies on AMR published which covered the aspect of AMR in environment [25]. In this study, environmental isolates were most resistant to Nitrofurantoin (80%) followed by Furazolidonones (70%), Oxytetracycline (66.66%) and 56.67% each for Nalidixic acid, Clarithromycin & Co-trimoxazole. A systematic review and meta-analysis on global status of antimicrobial resistance among environmental isolates of *Vibrio cholera* O1/O139 also reported increased resistance to nalidixic acid, co-trimoxazole, furazolidone and tetracycline during the years 2000–2020 [34]. Similarly, high resistance was also seen against ampicillin (92.30%), cefazolin (88.46%), ceftazidime (73.0%), and fluoroquinolones (65.38%), in UTI isolates from tertiary care hospital in New Delhi [35].

There are several drivers of AMR in the environment like release of pharmaceutical waste waters and hospital effluents without adequate treatment, irrational use of antibiotics and use of antimicrobial in animals, use of biocides in agriculture, open defecation practices. Several workers reported varying concentration of drug resistant gram-negative bacteria from sacred rivers (Ganges and Yamuna) in northern India [36-37], *E. coli* isolates with 100% resistant to third generation cephalosporins in south Indian river Cauvery in Karnataka [38]. From Central India, 17% rate of E. coli resistant to third generation cephalosporin were found in groundwater and surface water used for drinking and recreational purposes[39], 7% in north India (Kashmir) [40], 50 per cent in east India (Sikkim) [41] and 100 per cent in south India (Hyderabad) [42]. In addition to aminoglycosides, it was found that *Klebsiella spp.*, *E. coli* and *Enterobacter spp.* were sensitive to fluoroquinoloes. Geetha *et al* (2020) from Tamil Nadu (India) reported 100% resistance to fluoroquinolones class among clinical *K. pneumonia* isolates [43]. The difference may be due to lower sample size in our study or number of antibiotics was also less in our case compared to the study by Geetha *et al* (2020).

In our study, one isolate of *Bacillus spp.* with XDR phenotype was recovered from a local street vended food Pani Puri. In the study area, it was observed that the dish washing was usually done in buckets and sometimes without soap due to non-availability of running water near the vending site and that might contribute for bacterial contamination. Although this study did not quantify the total number of pathogenic bacteria but presence of drug resistant pathogens in street vended food could pose a serious health concern to the consumers. Other sources of bacterial contamination of street vended Pani Puri could be use of soil contaminated water, cross contamination by unwashed hands, and use of raw vegetables. Similarly, a study from Nepal reported high loads of bacterial pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella spp.*, *Pseudomonas spp.*, *Bacillus spp.* in Pani Puri [44].

Ghimire *et al* (2021) also reported contamination of Pani Puri water with *Staphylococcus aureus*, *Escherichia coli* and *Salmonella spp.*[45]. Another studies from Bengaluru India also reported different bacterial pathogens from street vended Pani-Puri[46]. Pathogenic bacteria like *Escherichia coli, Klebsiella spp., Enterobactor spp., Bacillus spp., Enterococcus spp., Micrococcus tetragens,*

Salmonella paratyphi, *Shigella dysenteriae* and *Vibrio spp.* were detected from Odisha (India) [47]. In this study, the only isolate of *Enterococcus spp.* recovered from local ravine water near Parwanoo area of Himachal Pradesh found resistant to Netilin drug from aminoglycosides class, in addition to resistance to other antibiotic classes tested. However, the isolate was sensitive to other antibiotics (gentamicin, kanamycin, amikacin, tobramycin and streptomycin) from the same class. Similarly, lower resistance rate was reported for gentamicin among *Enterococcus faecalis* (13.3%) and *E. faecium* (18.9%) isolates from activated sludge wastewater treatment system [48].

In contrast to this, high level aminoglycoside resistance (HLAR) genes are widely disseminated among isolates of *Enterococci* from Chennai as reported by Padmasini *et al* (2014) [49]. In this study, XDR phenotype was more prevalent (73.68%) as compare to only MDR phenotype (26.32%) among recovered environmental isolates. This means drug resistance pattern is amplifying towards higher level of resistance, and dissemination of such highly resistant strains in different environmental niches is a matter of great concern. All MDR, XDR and MDS isolates have been statistically tested for comparative analysis of selective virulence factors by using Chi (χ^2) test. The expression of virulence factors studied had no significant relation with the resistant phenotype as the χ^2 (tabular) was more than the χ^2 (calculated) for the significance level 0.05 and degree of freedom [12]. One study from Himachal Pradesh (India) also observed no significant difference between MDR and MDS *P. aeruginosa* clinical isolates for expression of virulence traits [17]. Other studies have also reported no significant relation between expression of virulence traits and multi drug resistant phenotype [50-53].

Health care facilities are not the only place where resistance can originate and spread. The incidence of antibiotic resistance in the environment is a serious threat to public health. Now it's high time to implement affirmative action plan to prevent dissemination of MDR strains in the environment. The continuous surveillance and characterization of such strains in the hospital settings as well as in the communities therefore is essentially required. The determination of susceptibility patterns against different classes of antibiotics, investigation of the mechanisms of virulence and to study the drug resistance mechanisms and epidemiological studies are some of the very important aspects that are required to be studied.

CONCLUSION: It may conclude from the present study that multiple drug resistance (MDR) and extensively drug resistant (XDR) bacteria are prevalent among different environmental niches in the study area. This study investigated only aerobic mesophilic bacteria while anaerobic bacteria and fungal pathogens are also of equal importance but they were not included in this study. Antimicrobial resistance (AMR) in environmental settings can impose a significant public health problem as the paradigm is shifting towards higher level of resistance. Aminoglycosides class of drugs can be used in treating infections due to these organisms as evidenced in this study. Although, there was no statistically significant relation found between expression of virulence traits and resistant phenotype of the bacterial isolates, but they are often associated with emergence of multidrug resistant, extensive drug resistant and pan-drug resistant isolates and the leading cause of treatment failure. Potential drivers of AMR in the environment must be looked upon and strict adherence to biomedical waste management guidelines must be followed to contain the spread of such drug resistant strains in the natural environment.

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CONFLICTS OF INTEREST: Nil

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