SPREAD OF MULTIDRUG RESISTANCE THROUGH MOBILE GENETIC ELEMENTS IN DAIRY PRODUCTS

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ABSTRACT: Various food and water sources contain antibiotic resistant microbiota. In this study, the resistance rate of antibiotics in bacteria isolated from food and water sources was checked. Promising isolates were identified as *Bacillus safensis* (MK934485), *Pseudomonas putida* (MK934486), *Pseudomonas spp*. (MK934484), *Escherichia coli* (MW713177, MW692366), *Klebsiella spp*. (MW713441). Nine antibiotics were used to check resistance patterns of isolated strains. Resistance pattern observed high to low was Ampicillin (100%), Cefotaxime (82%), Ceftazidime (76%), Augmentin (52%), Cefixime (64%), Meropenem (17%). *Escherichia coli* showed resistance against Amoxicillin, Meropenem, Clarithromycin, Cefoxitin, and Cefuroxime. *Klebsiella spp.* were resistant against Cefuroxime, Cefoxitin, Clarithromycin, Meropenem and Amoxicillin. Strains showed MIC at 1000µg/ml of ampicillin. Gene *intI1* from *E. coli* was found to have conserved domains of integrase. Hence, precautionary measures are necessary to halt the progression of antibiotic resistance.

Keywords: *amp* gene, *intI11* gene, minimum inhibitory concentration (MIC), mobile genetic elements, plasmid

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INTRODUCTION

Antibiotics are the leading chemicals used to fight any type of infection caused by bacteria. Antibiotics are characteristic or manufactured compounds that can repress microbial development or straightforwardly eliminate microscopic organisms or parasites. Yet they highly adapt themselves from antibiotics by developing different methods inserting resistant genes in their genetic material. The bacteria which are resistant to the antibiotic have become one of the major threats to human health. The more we use antibiotics, the more the resistance is growing against them. The antibiotic resistant bacteria have become so common that even food, raw as well as processed, has been found to contain antibiotic resistant bacteria. Growth promoters widely use tetracycline. Furthermore, infections instigated by these pathogens are commonly associated with countless mortality rates and longer hospitalizations (Navon-Venezia, Kondratyeva, and Carattoli, 2017).

It has been found that even the non-pathogenic strain of *Escherichia coli*, which can be found in many foods, has become resistant to many antibiotics (Badri et al., 2017). A healthy person contains resistant bacteria in their normal flora of intestine and feces. These bacteria provide a huge reservoir for multidrug resistant bacteria (Somily et al., 2014). Over the years, resistance to cephalosporins among members of Enterobacteriaceae has increased mainly due to the spreading of Extended-spectrum β-Lactamases (ESBL) (Tripathi et al., 2014)

Propagation of antimicrobial resistance among bacterial populations is an increasing problem worldwide. The bacteria have the ability to acquire any DNA from the environment and then incorporate this DNA into their own DNA. Mutation as well as acquisition has been found responsible for giving rise to the resistant elements. The process of conjugation, however, facilitates the horizontal transfer of these genes and therefore, spreads resistance in the population. Once a bacterial strain has become resistant to an antibiotic, it cannot be treated by using that antibiotic at all. The genetic elements which are responsible for this antibiotic resistance include integrin class 1 and 2 (de Los Santos, Laviña, and Poey, 2021).

Mobile genetic elements (MGE) act as the keywords that dictate conjugation as well as transduction. Plasmids hold an important role in this process. The MGEs have been found responsible for giving rise to antibiotic resistance as well as pollutants. Moreover, they have also been found to be causing the horizontal gene transfer (HGT)(Zhao et al., 2019). Thus, there is no exaggeration in saying that MGEs have a vital role in bacterial catabolism. Sometimes, MGEs give rise to a completely modified metabolic pathway by horizontal gene transfer(Rodríguez-Beltrán, DelaFuente, Leon-Sampedro, MacLean, and San Millan, 2021).

Transposable elements are amongst the most important MGEs and can be classified into two major types i.e., Insertion sequence, which are the short sequences of DNA of size ranging from 0.7 to 2kb, and

Transposition elements, which are large sequences of DNA having size greater than 2kb. They don't have the genes responsible for transposition and regulation but for resistance against the stress of antibiotics and heavy metals(Babakhani and Oloomi, 2018).The genetic exchange of the bacterial genes responsible for antibiotic resistance occurs via plasmid DNA during the process of conjugation. The method of interest is an efficient one to determine the acquiring of resistance by bacteria in clinical settings and it is based upon the principles of conjugation. Transformation, which is the bacterial sexual process, helps transfer the resistant genes between the bacteria and the transformation may even transfer the genes of antibiotic resistance to other bacterial species as well for instance the resistance to penicillin in *N. meningitidis, S. pneumoniae*and *N. gonorrhoeae.* Each resistant strain has its own mechanism of resistance and therefore has a different target for example the proteins responsible for penicillin binding(L. Li et al., 2018).

MGEs are involved in the spread of antibiotic resistance by site specific recombination. So bacterial integrons capture genes and form a site-specific enzyme for recombination which is integrase. Gene cassettes are non-replicating dsDNA which comprise the movement of genes from one integron to another. Integrons contain *attI* site(**Error! Reference source not found.**). They cannot catalyze theirmovement due to the absence of functional genes for mobility. There are two DNA segments in integrons and these segments have different specified regions containing genes, inserted in the form of cassettes. PCR mapping of the integrons is a good strategy to study the plasmids containing the genes of resistance(Christaki, Marcou, and Tofarides, 2020).

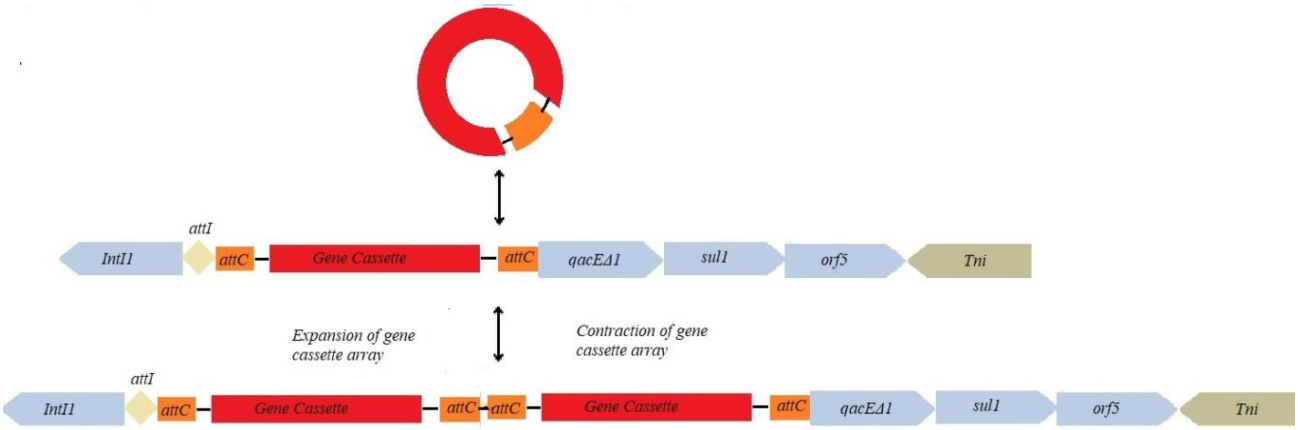

Figure 1: Mechanism of *IntI1* mediated gene cassette insertion in bacterial genome. Here 5' CS and 3' CS are conserved sequences, *IntI1*encodesintegrase, *attI* is the integration site of integron gene and also involved in site-specific recombination, qacEΔ1 is the quaternary ammonium compounds resistance, *sul1*encodesfor resistance to sulphonamides while orf5 isgene of unknown function. Recombination between *attI* and corresponding *attC* sites allow the insertion of gene cassettes in Integrons giving rise to cassette arrays.

MATERIALS AND METHODS

Sampling and microbial isolation: Different commonly available food products (i.e., ketchup, mayonnaise, dairy milk, canned milk, minced goat meat, chicken meat, raw eggs, carrot, cabbage, and spinach) and water samples (sewage and industrial water) were collected from Lahore (31°15−31°45'N and 74°01−74°39'E) in sterilized screw-capped bottles. Temperature, pH and odor of samples were noted at collection. Samples were processed for isolation of microbial floraby serial dilution method. Water samples were processed for calculation of CFU with or without antibiotic stress. From all samples, forty seven bacterial strains were isolated based on colony morphology and characterized morphologically, biochemically, and physiologically (James and Natalie, 2014).

Antimicrobial susceptibility testing: Disc diffusion method was used in this study to identify antibiotic resistance in isolated bacterial strains. Mueller-Hinton agar was utilized for susceptibility testing using different antibioticsas per standard i.e., Rifampicin (RP2) 2 µg, Meropenem (MEM10) 10µg, Levofloxacin (Lev1) 1µg, Amoxicillin-Clavulanic acid (Aug3) 3µg, Amoxicillin (A2) 2µg, Ampicillin (AP2) 2µg, Cefalexin (Cfx30) 30µg, Noreloxacin (Nor2) 2µg, Cefoxitin (Fox10) 10µg, Ciprofloxacin (Cip1) 1µg, Cefixime (CFM), Cefotaxime (CTX), Augmentin (AUG), Ceftazidime (CAZ) and Tetracycline (T1) 1µg.Antibiotic discs were applied and incubated for 24 hours at 37°C. The measurement of zone of inhibition was done according to Laboratory Standards Institute method (CLSI) 27th edition. Double disc assay was done using three antibiotics (30µg) i.e., Ceftazidime, Augmentin and Cefotaximeto see the effectiveness of any

antimicrobial agent. Lawn of bacterial strains was made on Mueller-Hinton agar plate. Augmentin disc was placed in the middle while the other two were placed on the sides of plate.

Minimum inhibition concentration: Stock solutions of antibiotics were used for evaluating the minimum concentration of antibiotics resistance towards the bacterial strains. Stock solutions were prepared for Oxytetracycline, Tetracycline, Streptomycin and Ampicillin and added to the N-agar media in increasing concentrations. Bacterial strains were streaked on the plates and plates were incubated for 24 hours at 37°C.

Genomic and Plasmid DNA isolation: Thermo Scientific DNA Miniprep kit (REF: k0503) and Thermo Scientific Gene JET Genomic DNA purification kit (REF: K0721) were used for isolation of genomic DNA while Thermo Scientific Plasmid Miniprep kit (REF: k0503) was used to isolate plasmid DNAfrom antibiotic resistant bacterial strains, according to the guidelines of the manufacturer. Gel Electrophoresis was done to check the bands of plasmids.

Phylogenetic analysis of 16s rRNAsequences: Antibiotic resistant bacterial strains were identified via 16S rRNAsequences using Sanger dideoxy sequencing. Chromas Pro 2.6.5 software was used to convert reverse primer to a complementary sequence and consensus Sequences were obtained by aligning forward and reverse sequences by using Cap3 software. Nucleotide Blast was used to examine sequence for all-out/maximum homology against GenBank whileMEGA4 was used to construct phylogenetic trees by neighbor-joining method (Kumar, Stecher, Li, Knyaz, and Tamura, 2018). All sequences were submitted to NCBI GenBank. Conserved domain database (CDD) to identify conserved domains.

Amplification of *intI1* and *Amp* genes: Primer for integron gene IntI1-F TCTCGGGTAACATCAAGG and IntI1-R AGGAGATCCGAAGACCTC were used for amplification of antibiotic resistant Integron*intI1*through PCR (Shamsizadeh et al., 2021). While gene *Amp* was performed by using primers Amp-F CATATGCTTAATCAGTGAGGCACCT and Amp-R GAATTCAGTATTCAACATTTCCGTGTCG with annealing temperature 44°C for 45 seconds. at 44°C, followed by the extension at 72°C of 45 seconds. The amplified product was purified by using Fermentas Gene JET PCR purification kit (REF: K0691) and sent for sequencing. NCBI Blast was used to find homology and MEGA4was used to construct phylogenetic trees by neighbor-joining method. Conserved domain database (CDD) was used to identify conserved domains.

RESULTS

Bacterial isolation and characterization: Total forty-seven strains were isolated from foodand water samples. These strains were characterized morphologically and biochemically. Selected isolated strains were selected via 16S rRNA sequencing and accession numbers of strains are provided in table.

Table 1 Accession Number of 16S rRNA sequences from strains that showed presence of intI1 gene.

Strain name	Organism Name	Accession Numbers
ST1	*Pseudomonas spp.*	MK934484
ST2	*Bacillus safensis*	MK934485
ST3	*Pseudomonas putida*	MK934486
SW	*Escherichia coli*	MW713177
S5	*Escherichia coli*	MW692366
T36	*Klebsiella spp.*	MW713441

Antibiotic disc results: Isolated strains showed different patterns i.e., either they remained susceptible or resistant towards used antibiotics (**Error! Reference source not found.**2) and (**Error! Reference source not found.**). Antibiotics showed more resistance as compared to susceptibility. About 100% isolates showed resistance to amoxicillin and meropenem, while 80% showed resistance to Clarithromycin. Among the isolates from food samples, widespread resistance was shown against Penicillin, Cephalosporins and Carbapenems.

Minimum inhibition concentration (MIC); Minimum inhibition concentration of antibiotics; Tetracycline, Streptomycin, Oxytetracycline and Ampicillin were calculated (Figure ***3***3). Ampicillin and streptomycin showed highest MIC, which was 1000µg/ml. Tetracycline showed MIC at 300µg/ml. MIC of Ampicillin was very high as compared to Oxytetracycline. Oxytetracycline showed MIC at 10µg/ml. For Streptomycin approximately 40% bacterial isolates showed MIC at 1000µg/ml. In this case, 60% bacteria showed sensitivity at 1000µg/ml. MIC of Tetracycline was 100µg/ml and recorded by only 20% strains (Table 3).

Table 2. Resistance and Susceiptibility showed by isolated strains.

Antibiotics	Resistant Strains	Susceptible Strains
Amoxicillin (A2)	S18, S34, S21, S38, S16, SW, S11, TW, S32, T36	-
Meropenem (MEM10)	S18, S34, S21, S38, S16, SW, S11, TW, S32, T36	-
Clarithromycin (CLA2)	S18, S34, S21, S16, SW, S11, TW, T36	38, 32
Cefoxitin (FOX10)	S18, S34, S21, S38, S16, SW, S11, TW, S32, T36	-
Ciprofloxacin (CIP1)	S34	11, 38, 36, SW, 32, TW, 16, 18, 21
Chloramphenicol (C10)	S34, S38, TW	11, 36, SW, 32, 16, 18, 21
Cefuroxime (CXM5)	S18, S34, S21, S38, S16, SW, S11, TW, S32, T36	-
Levofloxacin (LEV1)	S34	11, 38, 36, SW, 32, TW, 16, 18, 21
ColistinSulphate (CO10)	S34, S38, S11, S32	36, SW, TW, 16, 18, 21

Sensitivity to Colistin Sulphate showed by six bacteria (T36, SW, TW, S16, S18, and S21) (Figure). Bacterial strains S11, T36, SW, S32, S16, S18, and S21 with variable zone of inhibitions showed sensitivity to Chloramphenicol. Bacteria S11, S38, T36, SW, S32, TW, S16, S18, and S21 showed Levofloxacin sensitivity. Strains S11, S38, T36, SW, S32, TW, S16, S18, S21 showed Ciprofloxacin sensitivity.

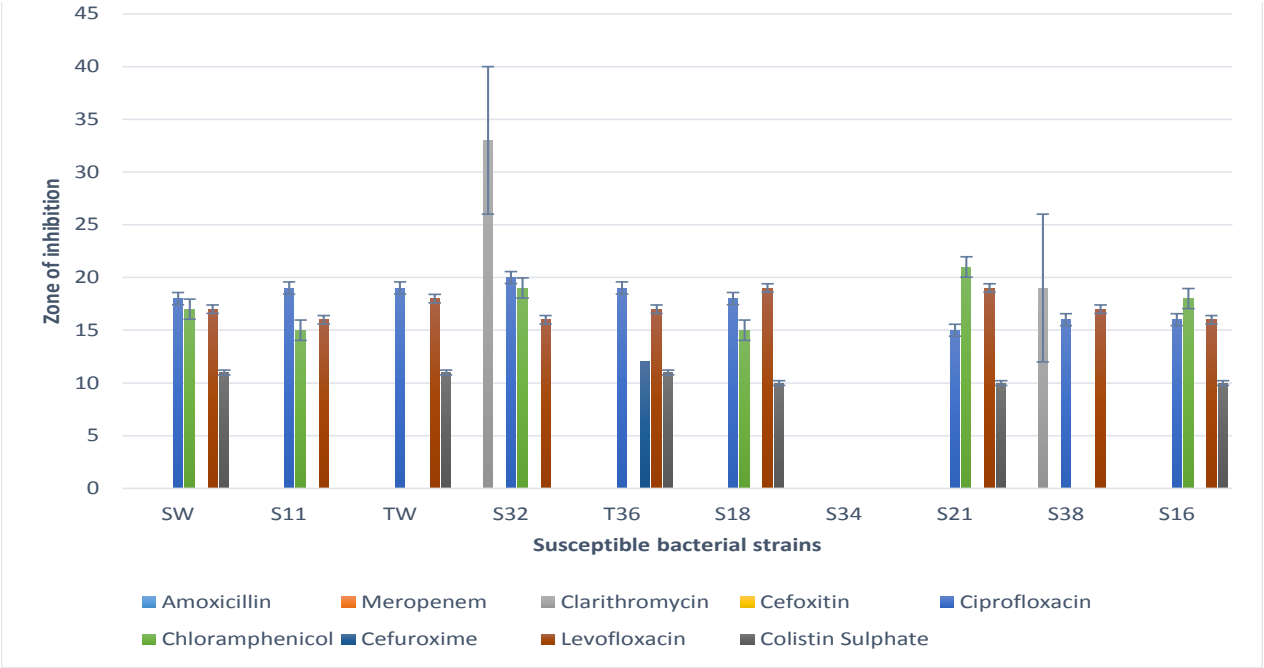

Figure 2: Sensitivity profiling of Bacterial isolates with different antibiotics with inhibition zone.

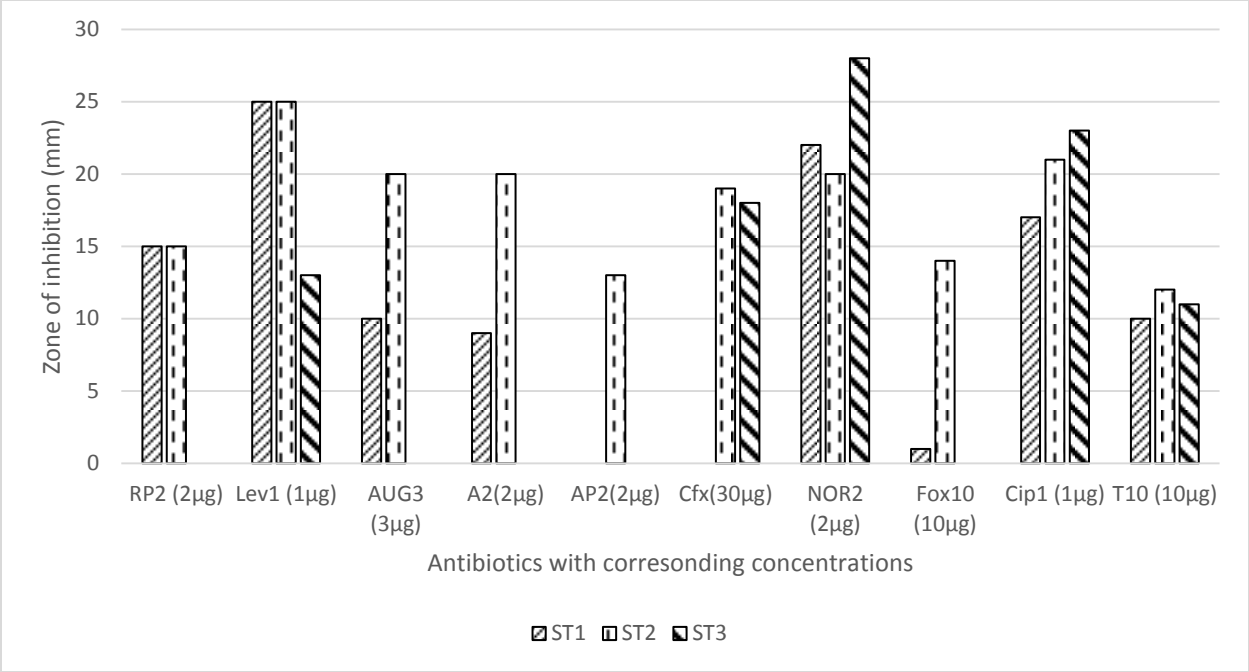

Figure 3 Inhibitory zones showed by isolates with different antibiotic discs. Zone of inhibition of the strains, which were sensitive and did not show resistant gene. These zones of inhibition were measured in mm.

DNA Isolation and Amplification of *intI1*gene: Genomic and plasmid DNA were isolated. Plasmids were only isolated in three strains; ST2, ST3, and ST19 **(Error! Reference source not found.**4a**).** ST2 showed 1500bp band. ST3 and ST19 showed 6000bp band. This shows the presence of the plasmid in the resistant strains. PCR method was used to amplify the DNA for i*ntI1*(class 1 integron of mobile genetic element; responsible for the spreading of resistant genes) and Amp genes. Amplified *intI1* gene of500bp was observed in strain ST3 along with a light band at 250bp (**Error! Reference source not found.**4b).Amplified *intI1* gene of 250bp was observed in strain SW1 along with a light band at 250bp (**Error! Reference source not found.**4c). For gene (Amp Table 3), band was observed at 850bpin strain T36 (**Error! Reference source not found.**4d). Search against conserved domain database showed presence of DNA BRE C super family conserved domains.

Table 3: Resistance showed by strains with the presence of mobile genetic element

Antibiotics	% Of Strains showing resistance	Strains containing gene as a mobile genetic element
Rifampicin	50%	ST3, ST15, ST20, ST16, ST19
Levofloxacin	10%	ST10
Amoxicillin-Clavulanic acid	40%	ST3, ST15, ST20, ST19
Amoxicillin	40%	ST3, ST15, ST20, ST19
Ampicillin	60%	ST3, ST9, ST15, ST20, ST1, ST19
Cefalexin	70%	ST9, ST15, ST20, ST10, ST1, ST16, ST19
Noreloxacin	-	-
Cefoxitin	40%	ST9, ST20, ST10, ST19
Ciprofloxacin	-	-
Tetracycline	-	-

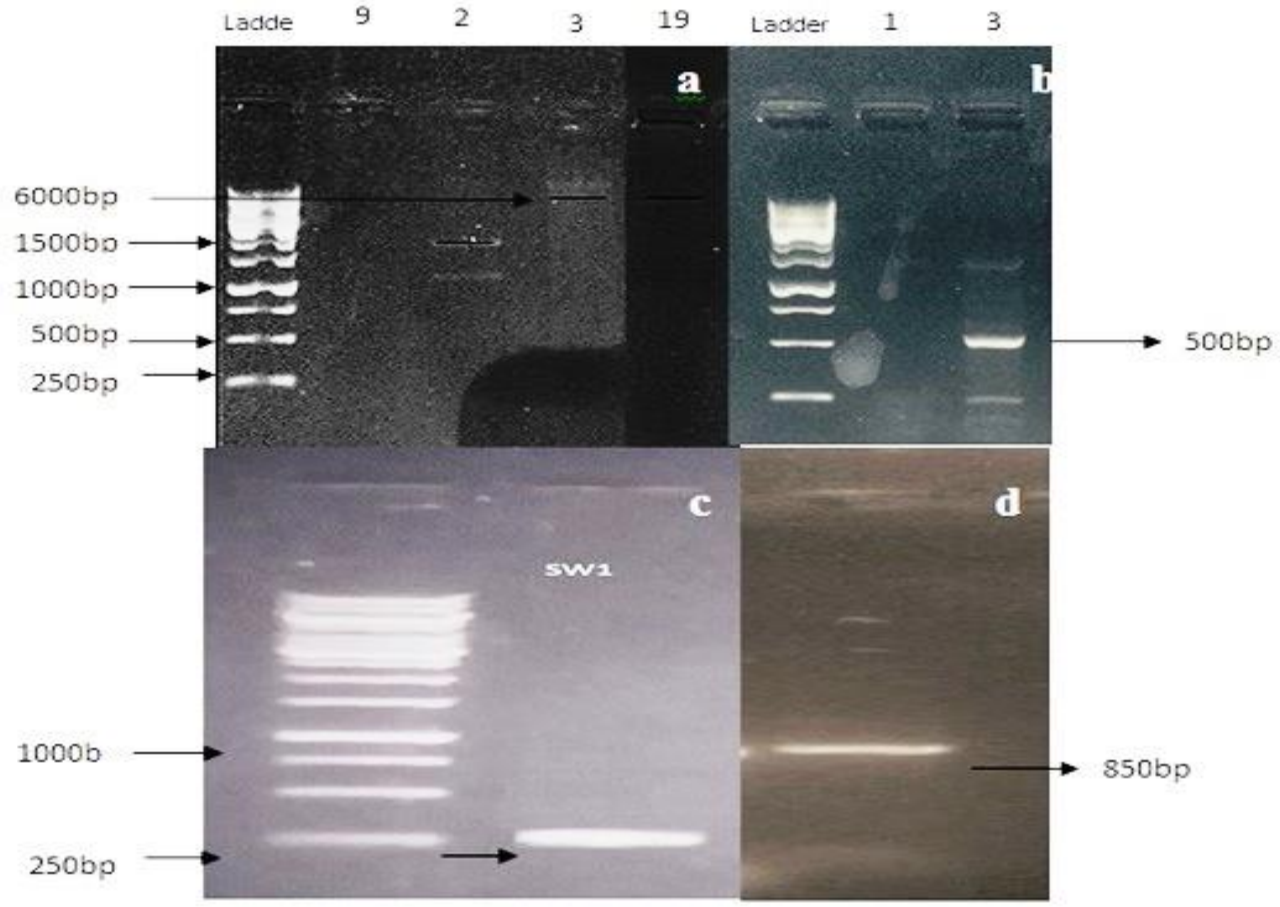

Figure 4: Results of presence of class 1 integron intI1 gene. (a) Plasmid isolation of resistant strains. ST2 showed 1500bp band. ST3 and ST19 showed 6000bp band. (b) Amplification of intI1 gene which shows the presence of class 1 integron at 500bp. (c) Amplification of intI1 gene which shows the presence of class 1 integron at 250bp. (b) Amplification of amp gene which shows band of 850bp.

DISCUSSION

In this study, screening for antibiotic resistant bacteria in water and food samples, shed some light on the serious problem of the common presence of antibiotic resistant bacteria in food items Bacterial pathogens were mainly isolated from sewage water. Gram negative and gram positive were further identified by different biochemical test. *Escherichia coli, Klebsiella spp.* and *pseudomonas spp.* were identified from gram negative bacterial strains. *Staphylococcus aureus, Bacillus spp.* and other gram-positive bacteria were also isolated. Human beings, on daily basis, are consuming an alarming amount of antibiotic resistant bacteria via food items and these bacteria include *Pseudomonas sp.* and *Streptococcus sp.* etc. Most of the recent studies involve the spreading of antibiotic resistant genes from different food sample. Approximately 10^2-10^7 CFU of antibiotic resistant bacteria has been reported in one gram ready-to-eat food sample (Caniça, Manageiro, Abriouel, Moran-Gilad, and Franz, 2019). This increase is mostly due to an increased prevalence of ESBL producing Enterobacteriaceae and an increase in using last resort antimicrobial drugs. ESBL producing Enterobacteriaceae isolates have moved from the hospital to the community and the environment in recent years. ESBL producing Enterobacteriaceae have been found on various food sources in the community, and a study from India reported that a large percentage of samples of tap water are infected with carbapenems blaNDM-1 producing organisms. In a recent study, reported CFU for industrial waste water sample with dilution factor of 10^{-2} was $2.6x10^5$ CFU/ml (without antibiotic) and with antibiotics was $3.2x10^5$ CFU/ml (with ampicillin), $5.2x10^5$CFU/ml (with tetracycline) as in (Jinmei Li, Phulpoto, Zhang, and Yu, 2021). Studies on this subject have been done mostly in developed countries, while most of ESBL expressing bacteria are reported in Asia, Africa and Middle East (Walsh, Weeks, Livermore, and Toleman, 2011). It was indicated by the results of this study that 41% isolates were positive for ESBL producing *E. coli* and of them

14.2% isolates were positive for *IntI1* and *Amp* gene. Owing to the presence of *E. coli* presence in intestinal tract, it can be considered as fecal contamination in food samples causing diarrhea. *E. coli* possesses considerable antibiotic resistance not just because it is a common gram negative pathogen in humans, but because it is the common culprit for most urinary tract infections and is the cause of most community and hospital acquired bacterial infections. Resistant *E. coli* strains can also transfer antibiotic resistance determinants to other strains of *E. coli* as well as to other bacteria in the gastrointestinal tract and other organisms to obtain resistance.

In this study all bacterial strains showed sensitivity against beta-lactam antibiotics, quinolone, polymyxin, aminoglycoside, cephalosporin second generation cephamycin, macrolides, fluoroquinolones, streptomycin. Strains ST10, ST16, ST19 showed resistance against Rp, Ap, and Aug. Ciprofloxacin resistance was shown in 90% isolates. Similar results were reported by Khan *et al.* (Khan, Beattie, and Knapp, 2016). All antibiotics showed 100% sensitivity for meropenem and Cefuroxime. 80% of bacterial isolates were resistant to clarithromycin. A study in North Ethiopia reported 90% ciprofloxacin and Levofloxacin resistance in the strains (Shiferaw, Gelaw, Assefa, Assefa, and Addis, 2015). 70% of isolates were resistant to chloramphenicol and 60% were resistant to Colistin sulphate. Similar results were reported in a study conducted by Jun Li *et al.*(Jun Li et al., 2019). Strains ST3, ST1, ST16, and ST19 showed resistance against Cefalexin and Cefoxitin. Cefalexin observed the highest resistance. Wang et al. 2019 reported tetra- and penta-multi-drug resistance patterns in *Salmonella typhimurium* against ampicillin, sulfonamides, chloramphenicol, tetracycline and streptomycin (Wang et al., 2019). Ampicillin showed 60% resistance among all antibiotics. All isolates of *E. coli* were resistant to ampicillin. However, previous studies have found that ampicillin was the least effective among the tested antimicrobials against *E. coli* (Ibrahim, Bilal, and Hamid, 2012). A study of milk samples in Sudan has reported a finding where 98 percent of the cephalosporin resistant *E. coli* isolates were also ampicillin resistant. Our results suggest that most of the *E. coli* isolates were susceptible to meropenem. Resistance of *E. coli* isolates to cefotaxime (82 %) was observed. Resistance to cefotaxime emerged, and resistance to β lactam antibiotics became the main mode of resistance. Most of this resistance was mediated by ESBLs, which confer resistance to newer β-lactam agents like ceftazidime, cefixime and augmentin (Pitart et al., 2015). In this study, all isolates were higher resistance than the study conducted in North America. However, there is concern about increasing resistance to third generation cephalosporins such as cefotaxime, ceftazidime, cefixime for example, among Enterobacteriaceae (Yemm et al., 2018). Different sources in the community, such as cattle, chickens, raw milk, vegetables, have yielded ESBL producing Enterobacteriaceae. *E. coli* isolated from fecal samples have been reported to be multi drug resistant among 27% of isolates (Eltai et al., 2018).

MIC of three antibiotics were detected in this study. All of the isolated strains had ampicillin as the only antibiotic with MIC ≥1000µg/ml. This shows that ampicillin is that antibiotic which gives resistance in highest amount too. This proves that ampicillin is the most resistant that antibiotic too. One of the study observed MIC of ampicillin ranging 700 µg/ml whereas, our study showed MIC of > 1000µg/ml for *E.coli*, *Klebsiella spp. and Pseudomonas spp*. (Khatoon, Alam, Khan, Raza, and Sardar, 2019). Their MIC of tetracycline in SW (E.coli) and in TW strain was about 300µg/ml. E.coli isolated from water also showed similar results in a study from (Aslan, Cole, Bhattacharya, and Oyibo, 2018).

Mobile integrons are very crucial in spreading the gene cassettes from one strain of the bacteria to another. Integrons, which belong to class 1, can be found both in plasmids as well as the transposons (Shamsizadeh et al., 2021). Class 1 Integrons are the major player in antibiotic resistance by using them to capture gene cassettes from a huge database of antibiotics resistance genes. Also, they contribute to the spreading of resistance in an environment through presence on various types of mobile genetic elements (Zhu et al., 2017). Pathogenic bacteria commonly carry class 1 integrons. In fact, aminoglycoside and trimethoprim resistance is not due to these integrated genes in the integron, but the gene cassettes themselves being integrated in the integron. This wonderful association of ampicillin and integrons is demonstrated by different analysis. Although this association is insignificant relative to other antibiotics. The objective of the project is to investigate *intI1* and Amp resistance gene to investigate a transposable element transferring resistance genes between bacterial species in environmental water. The results demonstrate presence of transposable element in the microbiota of environmental water for molecular characterization of class 1 integron *intI1* and Amp resistance gene. The Amp gene was also detected as confirming that the resistance genes were transposed from one bacterial genome to another due to transposons. In a study, the class 1 integron, with *intI1* gene detected was present (Lavakhamseh et al., 2016). Bioinformatics analysis of *E. coli* confirmed the presence of transposable element. Sequenced gene shows the conserved domain of this *int* gene, indicating that this *int* gene is a sequence of integrase for site specific recombination. These findings support the ability of *intI1* gene to provide the role of the primary mobile genetic element involved in the horizontal transfer of antibiotic resistance genes between different bacterial species. Additionally, this study also

highlights the presence of Integrons conferring antibiotic resistance in *E. coli* sources from various environments.

Conclusion: In this study, antibiotic resistant bacteria were isolated form different food and water samples that contained transposable elements i.e., class I integron genes *intI1* and *Amp*. This study showed that rapid spread of antibiotic resistance is linked to mobile genetics elements. In addition, health authorities should take precautionary measures to control the spread of resistance, otherwise the results can be devastating.

Conflict of Interest: There is No conflict of interest between the Authors.

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