COMBINED EFFECT OF ANTIBIOTICS AND HEAVY METALS ON *E. COLI* ISOLATED FROM CLINICAL, SEWAGE AND POULTRY SOURCE

Lishba Zaman, Shomaila Sikandar, Imran Afzal

Department of Biology, LGU, Lahore, Pakistan

KEYWORDS	ABSTRACT
Multidrug Resistant, Heavy Metal, Poultry, Sewage and Clinical Sources	The rising threat of antibiotic resistance limits usefulness of presently employed antibiotics, where the heavy metals are known to play an important role in spread of this resistance. Present study investigated prevalence of multi-drug resistant, Colistin resistant and heavy metal resistant *E. coli* in clinical, sewage and poultry sources. Impact of heavy metals on antibiotics susceptibility pattern was analyzed. Antimicrobial susceptibility testing showed significant difference among the poultry, clinical and sewage isolates of *E. coli* ($p<0.05$) ($n=7$). Most frequently encountered form of resistance was observed against third generation antibiotics Ceftaxidime and Cefotaxime. All the isolates were found to be multi heavy metal resistant. Effect of heavy metals and antibiotics combination on the growth of nine MDR*E. coli* illustrated highest synergism for Gentamycin with four heavy metals combined, whereas only Ciprofloxacin exhibited antagonistic effect. This study concludes that occurrence and co-occurrence of heavy metals and antibiotics may induce & spread antibiotic resistance among various microbial species, threatening public health and ecological safety. Better understanding is needed for controlling the antibiotic resistance in bacteria.

INTRODUCTION

The antimicrobial resistance is one of the most serious global threats to human health in the Twenty-first century and responsible for rising incidence of both devastating and fatal diseases (McKenna 2013). The world health organization describes it as a global crisis and an imminent catastrophe of a return to the pre-antibiotic era (WHO, 2015). The increasing trend of resistance is closely linked with the misuse and over use of antibiotics in humans, animals and agriculture (Levy & Marshall 2004). Environmental bacteria like *Escherichia coli* have association with antibiotic-resistant gene, transfer these genes into normal flora of human and animal, exert strong selective pressure for the rise and spread of resistance (Kinge, Ateba & Kawadza, 2010; Alves, Pereira, Araújo, Castro, Correia & Henriques, 2014). The occurrence of antibiotics in environment stimulates the selection of bacteria resistant to antibiotics outside and also inside the clinical settings (Yang & Carlson 2003; Kummerer, 2009; Zhang, Zhang & Fang, 2009).

The studies in European and non-European countries illustrates remarkable number of antibiotic-resistant bacteria have shown in hospital and urban wastewater and sewage sludge. In addition, percentage of antibiotic-resistant bacteria in raw sewage and as well as in the emission of treatment plants, has tremendously increased in recent years (Goni, Capdepuy, Raymond, Quentin & Caumette, 1999; Reinthaler, Posch, Feierl, Wüst, Haas, Ruckenbauer & Marth, 2003; Silva, Castillo, Callejas, López & Olmos, 2006, Yang, Lin, Liao, Yeh, Chang, Tang & Liou, 2009). In humans and animals antibiotics are used for therapeutic purpose to control the bacterial infection and integrated at sub therapeutic dosages in feed of commercial livestock and poultry to enhance the growth. As a result the use of antibiotics intensify selection of resistant bacteria more than that of its therapeutic use against the clinical disease (Bogaard London, Driessen, Stobberingh, 2001), and this practice might add to resistance of antimicrobial agent in humans acquired through the human food chain (Witte, 1998).

The heavy metals and antibiotics are types of common pollutants of the environment from discharge and industrial activities, as well as both are harmful to ecological safety and public health (Zhu, Johnson, Su, Qiao, Guo, Stedtfeld & Tiedje, 2013). Their individual effect has been checked on organisms and environment almost for thirty years (Chiang, Santos, Ghyselbrecht, Cappuyns, Martens, Gerven & Meesschaert, 2012), However, the limited work has been done on the combined effect of antibiotics and heavy metals on the growth and antibiotic susceptibility of bacteria (Zhou, Xu, Xu, Zhang, X & Du, 2015). In natural environments, heavy metals and antibiotics co-exists play very important role in occurrence and spreading of bacterial resistance towards antibiotics (Austin, Wright, Stepanauskas & McArthur, 2006; Huang, Yu, Chen, Zhi, Yao, Liu & Liu, 2017) afterward a low concentration and continued exposure to these two types of common environmental pollutants (Filali, Taoufik, Zeroual, Dzairi, Talbi & Blaghen, 2000).

The efflux pumps, spontaneous chromosomal mutations and the conjugative plasmids are considered to be the thegeneral bacterial antibiotics resistance mechanisms, which can likewise elucidate co-resistance of bacteria to both antibiotics and metals in environment (Wang, Leung, Qian & Gu, 2006, Akinbowale, Peng, Grant & Barton, 2007). Thus, the co-existence of antibiotics and heavy metals may seriously threaten the ecological security, human health andenvironmentbecause of stress of heavy metals on microbial resistance to antibiotics (Xu, Ruan, Hou, Zhao, Zheng, Zhou & Yuan, 2014). So it is very important to illustrate the influence of antibiotics and heavy metal in deliberating resistance in bacteria (Zhou et al., 2015). The present work was performed to study the prevalence of multi drug resistant *E. coli* collected from different sources, and to characterize *E. coli* resistance against last resort antibiotic, Colistin. Moreover, resistance of these bacteria against heavy metals and co-occurrence of the heavy metals resistance and the antibiotic resistance was also studied.

MATERIALS AND METHODS

Sample Collection

Sampling was carried out from sewage, poultry and clinical sources. Sewage samples were collected from sewage system of Township, Bhatta chowk and Shahdra Lahore, Pakistan. Sterile glass bottles were used to collect sewage water. Six poultry samples were collected from three different Poultry shops (two from each). All the samples were obtained from chicken gut. Total twenty-four isolates of *E. coli* were collected from Microbiology Lab of Combined Military Hospital (CMH) Lahore, Pakistan.

Isolation and Identification

All the samples were processed by inoculating specimen on EMB media for the isolation of *E. coli*. Inoculated plates were incubated at 37°C for 24 hours. The isolated bacterial colonies produces green metallic sheen were selected and initially identified using Gram staining. Further confirmation and characterization of *E. coli* was done by the biochemical test according to Standard Bacteriological procedures (Cappuccino & Sherman, 2002). The biochemical tests applied were Catalase test and IMViC.

Antibiotic Sensitivity

To determine antibiotic susceptibility pattern of *E. coli* isolated from three different sources Kirby Bauer disc diffusion method on Muller Hinton Agar (MHA) was done by using the clinical and laboratory standard institute (CLSI) recommended methods and interpretative criteria (CLSI, 2016) to screen the Colistin resistant and multidrug resistant (MDR) *E. coli*. 10 commercially prepared antibiotics belonging to six different classes of antibiotics were used in this study. The tested antibiotics with their respective classes are in Table I.

Table I Antibiotics with their respective Classes (CLSI, 2016)

Classes of antibiotics	Antibiotics
Polymixin	Colistin (CT) 10 µg
Cephams	Ceftazidime (CAZ) 30 µg Cefotaxime (CTX) 30 µg
Quinolones and Flouroquinolones	Ciprofloxacin (CIP) 5 µg Norfloxacin (NOR) 10 µg
Aminoglycosides	Neomycin (N) 30 µg Gentamycin (CN) 10 µg Amikacin (AK) 30 µg
Nitrofurans	Nitrofurantoin (F) 300 µg
Folate pathway inhibitor	Trimethoprim (TMP) 5 µg

The inhibition zone diameter in millimeters was measured. The breakpoint used for each antimicrobial agent to categorize isolates as resistant, intermediate or sensitivefollowed the CLSI guidelines (CLSI, 2016).

Screening of Heavy Metal Resistant Strains of *E. coli*

For screening of heavy metal and multi heavy metal tolerant bacteria stock solution of four heavy metal salts potassium di chromate ($K_2Cr_2O_7$), lead nitrate ($Pb(NO_3)_2$), cadmium sulfate ($CdSO_4$), and sodium arsenite ($NaAsO_2$) were prepared. The nutrient agar was sterilized at 121°C for 15 min and allowed to cool 40-45 °C. Then the metals from stock solution were added to the nutrient agar at different concentrations (50 $ugmL^{-1}$, 100 $ugmL^{-1}$, 200 $ugmL^{-1}$ and 300 $ugmL^{-1}$) individually and all the four heavy metals salt was supplemented in two different concentrations (50 $ugmL^{-1}$and 100 $ugmL^{-1}$) according to calculated amount and transfer into petri plates. Point inoculation of all strains isolated from three sources was given and incubated at 37°C for 24 hours. Growth was observed after incubation.

Combined Effect of Heavy Metals and Antibiotics

To check the combined effect of heavy metal and ten antibiotics, nine strains were selected. All four heavy metal salts (Cd, Cr, Pb and As) were supplemented in Muller-Hinton media (50ug/mL each heavy metal salt) and bacterial suspension was spread on supplemented media. Ten antibiotics were placed on inoculated media and incubated at 37°C for 24 hours. Combined effect of antibiotics and heavy metals on the bacterial resistance was analyzed by measuring the inhibitory zone diameter and compared with the control (not supplemented with heavy metal salts).

Statistical Analysis

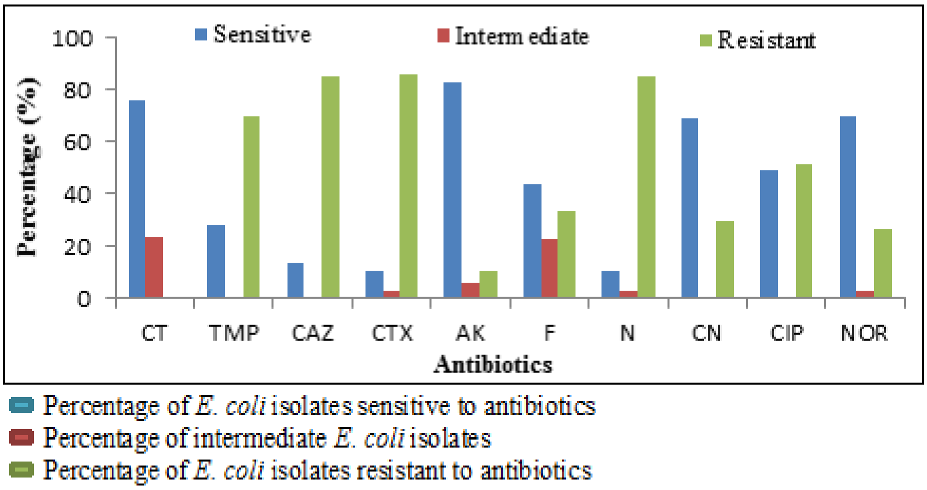
For statistical analysis, chi-square test was used to determine the significant differences among *E. coli* isolates from three sources regarding three levels of antibiotic susceptibility. Results were considered at 0.1% level of significance. To determine significant differences ($P<0.05$) among *E. coli* isolates from different sources based on size of inhibition zone, for each antibiotic, t-test were performed and results were considered at 5% significance level. Overall susceptibility of isolates against each antibiotic (on the basis of mean inhibitory zone diameter) was evaluating using 95% confidence interval.

RESULTS OF STUDY

The information about *E. coli* were isolated from sewage, clinical and poultry source which provide sufficient information in deciding the issue and to reach the conclusion. All the isolates produces metallic green sheen on EMB media appeared pink, rods, were found to be positive to Indole production test, Methyl red test and catalase test and negative to Vogues Proskeur test and Citrate utilization test. The overall susceptibility pattern of *E.*

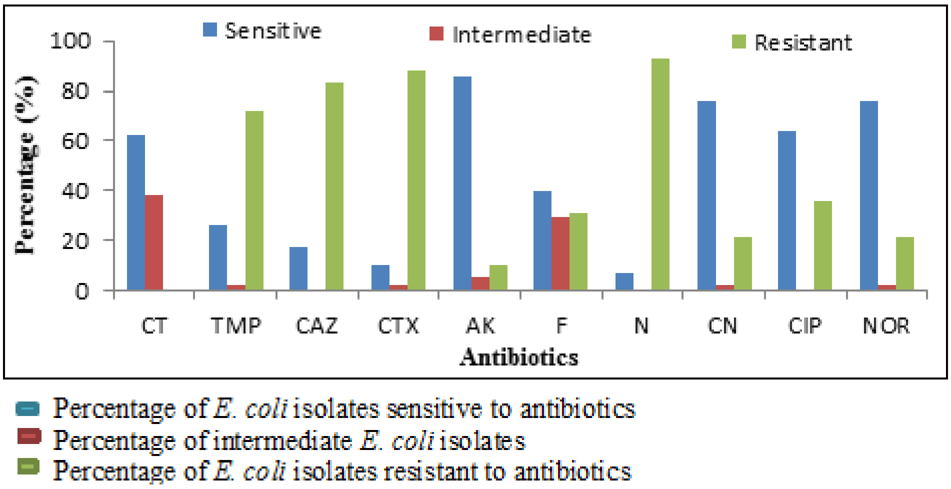
coli isolated form sewage, poultry and clinical sources is summarized in Figure 1. The result obtained in this study illustrates that highest percentage of resistance was observed against CAZ (86%), N (85%), CTX (85%), and TMP (70%). On the other hand highest degree of sensitivity to AK was detected. None of the isolate was found to be resistant to CT. Almost 24% strains gave intermediate zones against CT. CT Colistin; TMP Trimethoprim; CAZ Ceftazidime; CTX Cefotaxime; AK Amikacin; F Nitrofurantoin; N Neomycin; CN Gentamycin; CIP Ciprofloxacin; NOR Norfloxacin (Figure I).

Figure 1 Overall Antibiotic Susceptibility Pattern of *E. coli* isolated from Sewage, Poultry and Clinical Source against Ten Antibiotics.



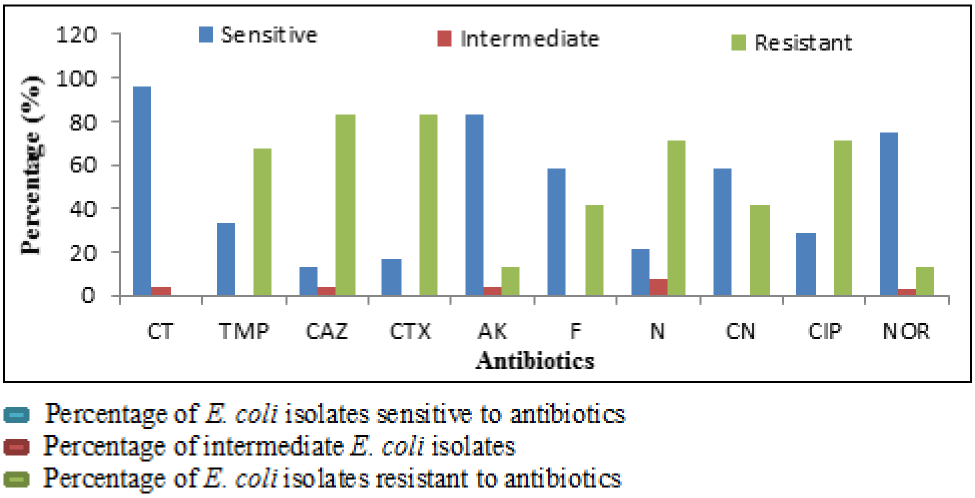
Based on 95% confidence interval, overall isolates in three sewage sources were found to be resistant to CAZ, TMP, CAZ, and N and sensitive to F, CT, AK, CN, NOR and CIP (Figure 2). CT Colistin; TMP Trimethoprim; CAZ Ceftazidime; CTX Cefotaxime; AK Amikacin; F Nitrofurantoin; N Neomycin; CN Gentamycin; CIP Ciprofloxacin; NOR Norfloxacin.

Figure 2 Antibiotic Susceptibility pattern of *E. coli* isolated from Sewage Source



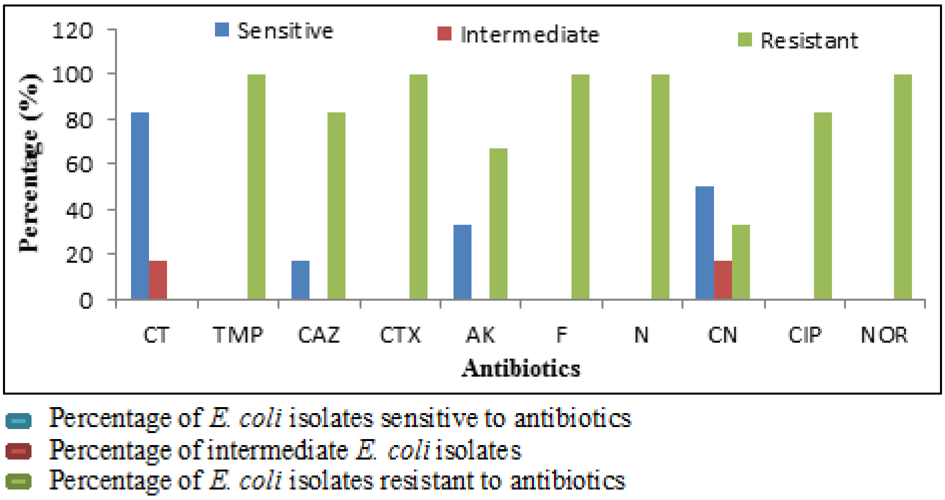
In case of clinical isolates most frequently encountered form of resistant was found against CTX, CAZ, CIP and TMP, and sensitive to F, CT, N, AK, CN and NOR, on the basis of 95% confidence interval (Figure 3). CT Colistin; TMP Trimethoprim; CAZ Ceftazidime; CTX Cefotaxime; AK Amikacin; F Nitrofurantoin; N Neomycin; CN Gentamycin; CIP Ciprofloxacin; NOR Norfloxacin.

Figure 3 Antibiotic Susceptibility Pattern of *E. coli* isolated from Clinical Source



Resistance was recorded for F, CAZ, TMP, N and NOR in poultry isolates. AK and Ceftazim gave intermediate zones however high sensitivity rate was recorded with CT and CN, based on 95% confidence interval (Figure 4). CT Colistin; TMP Trimethoprim; CAZ Ceftazidime; CTX Cefotaxime; AK Amikacin; F Nitrofurantoin; N Neomycin; CN Gentamycin; CIP Ciprofloxacin; NOR Norfloxacin.

Figure 4 Antibiotic Susceptibility Pattern of *E. coli* isolated from Poultry Source

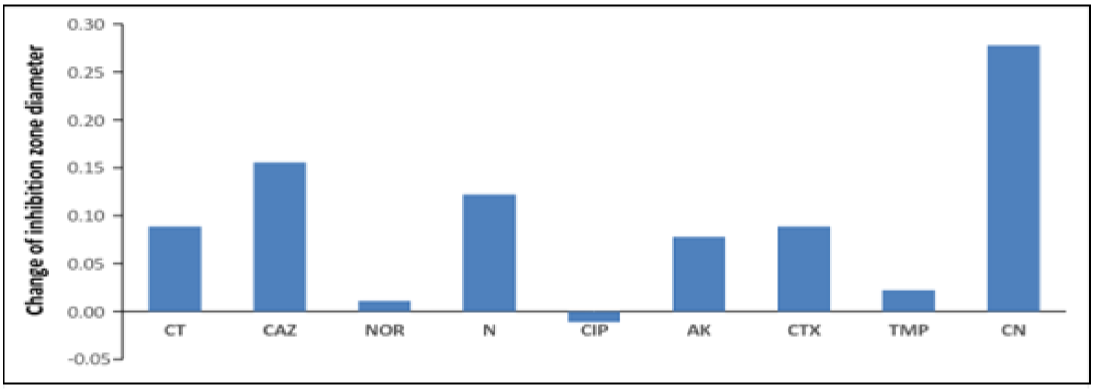


With regards to three sources, *E. coli* isolates in clinical source showed significantly (P<0.05) higher sensitivity against CAZ, CT, CTX and CIP but lower sensitivity against CIP than that of sewage isolates. When compared the susceptibility pattern among sewage and poultry *E. coli*, significant difference (P<0.05) in all tested antibiotics except CT, CN and CAZ was observed. Poultry isolates exhibited significant (P<0.05) resistance except CT, CN and CAZ with those of sewage isolates. It was demonstrated from statistical data that there was significant difference in susceptibility pattern among *E. coli* isolates of clinical and poultry sources except N, CN and CTX, clinical isolates were significantly sensitive to all antibiotics except N, CN and CTX in comparison with poultry isolates (P<0.05).

The data were statistically analyzed and expressed that there was highest significant difference (P<0.001) in antibiotic susceptibility among sewage, clinical and poultry isolates of *E. coli*. Multidrug resistance rates of poultry isolates of *E. coli* was significantly greater as compared to clinical and sewage isolates. Almost all the *E. coli* isolates of poultry were MDR. In addition, 86% of *E. coli* isolates of clinical source were multiple drug resistant, which was more than sewage isolates 69%. Antibiotic susceptibility testing revealed that 10 different antibiotic resistant patterns were formed. Maximum number of antibiotics to which strains of *E. coli* demonstrate resistance was 10 (14%) and at least resistant to one antimicrobial. Among all the strains only one strain showed no resistance towards any of the antibiotic. The most resistant pattern was shown by the combination of 4 antibiotics i-e CTX, TMP, N, and CAZ.

Heavy metal resistant *E. coli* isolates from three sources were screened for heavy metals resistance. Data showed that almost all the *E. coli* isolates gave the signs of growth on the agar plates supplemented with the heavy metals salt (Cr or Cd or Pb or As) at all the concentrations (50 $ugmL^{-1}$or 100 $ugmL^{-1}$ or 200 $ugmL^{-1}$ or 300 $ugmL^{-1}$) that clearly demonstrate that all isolates were resistant to all four heavy metals individually and all the isolates were also multi heavy metal resistant. Combined effect of the heavy metals and antibiotics (expressed as mean change of inhibition zone diameter) on growth of nine *E. coli* isolates illustrate highest synergism of CN together with 4 heavy metals whereas NOR and TMPshowed least synergism with four heavy metals. Only CIP exhibited antagonistic effect along heavy metals (Figure 5). There was highly significant ($P<0.001$) in proportion of nine *E. coli* tested for combined effect of antibiotics and heavy metals.

Figure 5 Combined effect of heavy metals and antibiotics (expressed as mean change of inhibition zone diameter) on growth of 9 *E. coli* isolates.



CT Colistin; TMP Trimethoprim; CAZ Ceftazidime; CTX Cefotaxime; AK Amikacin; F Nitrofurantoin; N Neomycin; CN Gentamycin; CIP Ciprofloxacin; NOR Norfloxacin.

DISCUSSION AND CONCLUSION

The present study was performed to assess and compare the antibiotic susceptibility and multi-drug resistance of *E. coli* isolated from different sources. The work also included the heavy metal tolerance of the isolates and effect of heavy metals on antibiotic susceptibility of the bacteria. The problem of increase and spread of antibiotic resistance in the bacteria begins since the day when antibiotics used for treatment of infection (Aminov & Mackie 2007). WHO describes the third largest threat to global public health in the 21st century is the emergence and spread of the antimicrobial resistant bacteria. Antibiotic resistance in *E. coli* is highly concerned hence *E. coli* is frequent gram negative pathogen in humans, the most important causative agent of urinary tract infection, community and hospital-acquired bacteremia (Salvadori, Coleman, Louie, McEwen & McGeer, 2004) and diarrhea (Kaper, Nataro & Mobley, 2004). Furthermore, resistant strains of *E. coli* have capability to transmit antibiotic resistant genes to other strains of *E. coli* as well as to other bacteria within gastrointestinal tract and acquired resistant genes from other organisms (Rawat & Nair, 2010).

Among most frequently encountered form of resistance in overall sewage isolates was resistance to N (93%), CTX (88%), CAZ (83%) and TMP (72%). On other hand, AK was recognized as most effective antibiotic as previously reported byPatoli (Patoli, Patoli & Mehraj, 2010). Notably, the percentage of resistance to CTX (88%), CAZ (83%) was almost similar as results obtained by Korzeniewska (Korzeniewska, Korzeniewska & Harnisz, 2013), but higher than previously reported by Chagas (Chagas et al., 2011) (48% and 15%) andPatoli (Patoli et al., 2010) (18% and 25%) respectively. Similarly, the present results differ from the work of Reinthaler (Reinthaler et al., 2003), who reported CTX, CAZ, AK and CN were 100% effective for E. coli isolates. Larger percentage of resistance to CIP (36%) and CN (21%) was observed in present study than those of found by Chagas, who ascertained, that 28% and 15% of isolates were resistant to CIP and CN, respectively. Consistent with the previous reports, the present data indicates that there is an increase in the resistance to the third generation Cephalosporins (CAZ & CTX) as reported by the WHO.

Higher percentage of sewage isolates gave intermediate zones against CT indicated that isolates turns toward resistance to CT due to its extensive use as a result of increased in number of MDR bacteria. Waste water is reservoir for antibiotic resistant bacteria of human origin. Henceforth, pattern of antibiotic resistance in waste water is the reflection of state of antibiotic pattern in the human population. The study of antibiotic resistant bacteria in the waste water is important to inquire an understanding as to which resistant bacteria are vulnerable than other to escape in environment, for instance environmental pathogens can be a source of new infection of humans (Reinthaler et al., 2013). However, *E. coli* is the most important causative agent of urinary tract infection in Pakistan and all over the world. *E. coli* are also important infectious agent in chickens, causing mortality in commercial poultry farms results in the economic losses(Ewers, Janben, Kießling, Philipp & Wieler, 2004). For the prevention of these economic losses antibiotics are the drug, used against *E. coli* infection and to increase the production efficiency (Angulo et al., 2000).

Data from present study revealed that AK and CT were the most effective drugs. Moreover maximum isolates showed resistance against third generation antibiotic like CTX and CAZ, indicated that these antibiotics lost their effectiveness and in future should not be used for the treatment of *E. coli* infection. Maximum number of sewage isolates showed intermediate zones against CT as compared to CT which suggested that in sewage there are various bacteria that transfers their genetic material (plasmid containing antibiotics resistance genes) into other bacteria and induce resistance in the sensitive bacteria as well. The residues of different antibiotics also prevail in sewage; as result pressure of antibiotics bacteria acquired resistance. The resistance of bacteria is taken similar, due to clustering of heavy metal and antibiotic resistant genes in plasmid (Xu et al., 2014), but detailed mechanism of cross-resistance is still unclear as there is limited study on combined effect antibiotics and heavy metals on bacterial resistance (Akiyama & Savin 2010). The results showed significant effect of antibiotic variety on mean inhibitory zone diameter of nine strains.

REFERANCES

Akinbowale, O. L., Peng, H., Grant, P., & Barton, M. D. (2007). Antibiotic and heavy metal resistance in motile aeromonads and pseudomonads from rainbow trout. *International Journal of Antimicrobial Agents*, 30(2):177-182.

Akiyama, T., & Savin, M. C. (2010). Populations of antibiotic-resistant coliform bacteria change rapidly in a wastewater effluent dominated stream. *Science of the Total Environment*, 408(24), 6192-6201.

Alves, M. S., Pereira, A., Araújo, S. M., Castro, B. B., Correia, A., & Henriques, I. (2014). Seawater is a reservoir of multi-resistant Escherichia coli, including strains hosting plasmid-mediated quinolones resistance and extended-spectrum β-Lactamases genes. *Frontiers in microbiology*, 5:426.

Aminov, R. I., & Mackie, R. I. (2007). Evolution and ecology of antibiotic resistance genes. FEMS Microbiology Letters. 271(2):147-161.

Angulo, F. J., Johnson, K. R., Tauxe, R. V., & Cohen, M. L. (2000). Origins and consequences of antimicrobial-resistant nontyphoidal Salmonella: implications for the use of fluoroquinolones in food animals. Microbial Drug Resistance. 6(1):77-83.

Austin, C., Wright, M. S., Stepanauskas, R., & McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends in Microbiology*. 14(4), 176-182.

Bogaard A. E, London, N., Driessen, C., Stobberingh, E. (2001). Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *Journal of Antimicrobial Chemotherapy*. 47, 763-771.

Cappuccino, J. G., & Sherman, N. (2002). Microbiology: A Laboratory Manual 6th edition San Francisco Benjamin Cummings Pearson Education.

Chagas, T., Seki, L.M., Cury, J.C., Oliveira, J.A., Dávila, A.M., Silva, D. M., & Asensi, M.D. (2011). Multiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital wastewater in Rio de Janeiro Brazil. *Journal of applied microbiology*. 111(3):572-581.

Chiang, Y. W., Santos, R. M., Ghyselbrecht, K., Cappuyns, V., Martens, J. A., Swennen, R., Gerven, T., & Meesschaert, B. (2012). Strategic selection of an optimal sorbent mixture for in-situ remediation of heavy metal contaminated sediments: framework and case study. *Journal of Environmental Management*, 105, 1-11.

Clinical and Laboratory Standards Institute 2016. Performance standards for antimicrobial susceptibility testing; twenty-sixth information supplement. Document M100-S26. Wayne, PA: CLSI.

Ewers, C., Janben, T., Kießling, S., Philipp, H. C., & Wieler, L. H. (2004). Molecular epidemiology of avian pathogenic *Escherichia coli* (APEC) isolated from colisepticemia in poultry. *Veterinary microbiology*. 104(1):91-101.

Filali, B. K., Taoufik, J., Zeroual, Y., Dzairi, F. Z., Talbi, M., & Blaghen, M. (2000). Waste water bacterial isolates resistant to heavy metals and antibiotics. *Current Microbiology*, 41(3), 151-156.

Goni, U. M., Capdepuy, M., Raymond, N., Quentin, C., & Caumette, P. (1999). Impact of an urban effluent on the bacterial community structure in the Arga River (Spain), with special reference to culturable Gram-negative rods. *Canadian journal of microbiology*, 45(10):826-832.

Huang, X., Yu, L., Chen, X., Zhi, C., Yao, X., Liu, Y., & Liu, J. H. (2017). High prevalence of Colistin resistance and *mcr-1* gene in *Escherichia coli* isolated from food animals in China. *Frontiers in Microbiology*. 8.

Kaper, J. B., Nataro, J. P., & Mobley, H. L. (2004). Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*. 2(2), 123.

Kinge, C. N., Ateba, N., & Kawadza, D. T. (2010). Antibiotic resistance profiles of Escherichia coli isolated from different water sources in the Mmabatho locality, North-West Province, South Africa: research letter. *South African Journal of Science*. 106(1-2):44-49.

Korzeniewska, E., Korzeniewska, A., & Harnisz, M. (2013). Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. *Ecotoxicology and environmental safety*, 91: 96-102.

Kummerer, K. (2009). Antibiotics in aquatic environment: A review–part I. *Chemosphere*. 75(4), 417-434.

Levy, S.B., & Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. Nature medicine. 10(12s): S122. https://doi.org/10.1038/nm1145

McKenna, M. (2013). The CDC Threat Report: We will soon be in the post-antibiotic era. The Wired.

Patoli, A. A., Patoli, B. B., & Mehraj, V. (2010). High prevalence of multi-drug resistant *Escherichia coli* in drinking water samples from Hyderabad. *Gomal Journal of Medical Sciences*. 8(1).

Rawat, D., & Nair, D. 2010. Extended-spectrum β-lactamases in Gram negative bacteria. *Journal of global infectious diseases*. 2(3), 263.

Reinthaler, F. F., Galler, H., Feierl, G., Haas, D., Leitner, E., Mascher, F., & Himmel, W. (2013). Resistance patterns of *Escherichia coli* isolated from sewage sludge in comparison with those isolated from human patients. *Journal of water and health*. 11(1):13-20.

Salvadori, M., Coleman, B. L., Louie, M., McEwen, S., & McGeer, A. (2004). Consumption of antimicrobial-resistant *Escherichia coli*-contaminated well water: Human Health Impact. *PSI Clinical Research*. 6-25.

Silva, J., Castillo, G., Callejas, L., López, H., & Olmos, J. (2006). Frequency of transferable multiple antibiotic resistances amongst coliform bacteria isolated from a treated sewage effluent in Antofagasta, Chile. *Electronic Journal of Biotechnology*, 9(5).

Wang, Y., Leung, P. C., Qian, P. Y., & Gu, J. D. (2006). Antibiotic resistance and plasmid profile of environmental isolates of Vibrio species from Mai Po Nature Reserve, Hong Kong. *Ecotoxicology*. 15(4), 371.

Witte, W. (1998). Medical consequences of antibiotic use in agriculture. *Science*. 279(5353), 996-997.

World Health Organization (WHO). (2015). Antimicrobial resistance. Available at http://www.who.int/mediacentre/factsheets/fs194/en/.

Xu, Y., Ruan, J., Hou, M., Zhao, X., Zheng, L., Zhou, S., & Yuan, B. (2014). Stress of five heavy metals on the resistance of isolates from swine wastewater to four antibiotics. *Hydrology: Current Research*. 5(3), 1.

Yang, C. M., Lin, M. F., Liao, P. C., Yeh, H. W., Chang, B. V., Tang, T. K., & Liou, M. L. (2009). Comparison of antimicrobial resistance patterns between clinical and sewage isolates in a regional hospital in Taiwan. *Letters in Applied Microbiology*. 48(5), 560-565.

Zhang, X. X., Zhang, T., & Fang, H. H. (2009). Antibiotic resistance genes in water environment. *Applied microbiology and biotechnology*, 82(3):397-414.

Zhou, Y., Xu, Y. B., Xu, J. X., Zhang, X. H., Xu, S. H., & Du, Q. P. (2015). Combined toxic effects of heavy metals and antibiotics on a Pseudomonas fluorescens strain ZY2 isolated from swine wastewater. *International Journal of Molecular Sciences*, 16(2):2839-2850.

Zhu, Y. G., Johnson, T. A., Su, J. Q., Qiao, M., Guo, G. X., Stedtfeld, R. D., & Tiedje, J. M. (2013). The diverse and abundant antibiotic resistance genes in the Chinese swine farms. *Proceedings of the National Academy of Sciences*, 110(9), 3435-3440.