ORIGINAL ARTICLE

FREQUENCY OF ANTIMICROBIAL RESISTANT GENES IN *E. COLI* STRAINS PRESENT IN EFFLUENTS OF PHARMACEUTICAL INDUSTRIES IN PESHAWAR

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Background: The intense use of antimicrobial agents for the treatment of different infections resulted in bulk manufacturing of antimicrobial drugs by the pharmaceutical industries. The wastewater of these industries contains traces of raw form of antimicrobial agents which drains into sewerage system of the area, the exposure to which results in the development of resistant microbes. The goal of our study was to determine the frequency of antimicrobial-resistance genes (ARGs) in *E. coli* present in effluents of pharmaceutical industries in Hayatabad Industrial State, Peshawar. **Methods:** In this qualitative analysis 5 wastewater samples each were obtained from effluents of 5 different pharmaceutical industries situated at Hayatabad Industrial Estate, Peshawar. *E. coli* in these effluents was cultured and identified through biochemical tests and Gram staining. DNA was extracted and ARGs such as *sul1*, *dfrA1*, *tetA*, *tetB* and *ermB* were analyzed through Polymerase Chain Reaction (PCR). The results were tabulated in Microsoft Excel 2016. **Results:** *E. coli* were detected in the samples with citrate utilization and indole and triple sugar iron tests. The effluents contained resistant strains of *E. coli* which have developed ARGs against major antibacterials such as *sul1* for sulfonamides (84%), *dfrA1* for trimethoprim (80%), *tetA* and *tetB* for tetracyclines (80%) while *ermB* for erythromycin (72%). **Conclusion:** The wastewater effluents of pharmaceutical industries may be one of the major sources of development of ARGs in microbes.

Keywords: sul1, dfrA1, tetA, tetB, ermB, antimicrobial resistance

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INTRODUCTION

The word 'antibiotic' was introduced by Selman Waksman in 1941 who was an American scientist, meaning a molecule which is produced by a microbe against other microbes.[1] Since the discovery of first antimicrobial agent, their use for the treatment of infections is tremendously increased. Besides the beneficial effects, there are several concerns over the uncontrolled use of more effective antibiotics in our society. The major emerging concern is the emergence of resistance in suspected microbes against the most potent and efficacious antibiotics causing a major threat to public health and safety.[2] Antibiotic resistance is defined as 'the development of resistance in microbes against the action of antimicrobial agents'.[3] The main goal of antibiotic discovery previously was focused only against Gram-positive bacterial diseases, but now Gram-negative bacterial diseases are more concerning in research field for new antibiotics development. Antimicrobial resistance is developing rapidly in Gram-negative bacteria mainly due to presence of jumping genes on bacterial plasmid DNA.[4] Several microbes develop resistance against many chemotherapeutic agents, known as 'MDR' (Multidrug Resistant). Typical examples are *Mycobacterium Tuberculosis*[5], *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* and *Enterobacteriaceae* which are becoming serious health problem now.[6] Antibiotic resistance develops mainly due to irrational prescribing, poor quality of drug formulation, water pollution and use of antibiotics in veterinary medicine and agriculture.[7] The bacteria acquire resistance against antibiotics through natural immunity, gene transfer mechanisms and plasmid-induced antimicrobial resistance.[8]

The presence of resistant bacteria in water ecosystems is an emerging problem worldwide. The studies performed on different water samples taken from different reservoirs like hospital wastewater, sewage-treatment plants, groundwater reservoirs and drinking water confirmed the presence of resistant species of *Enterococci*, *Enterobacteriaceae*, *Staphylococci* and other bacteria.[9] Even in the presence of water treatment plants, traces of antibiotics and resistant bacterial species could be very injurious to public health.[10] Patients treated with antibiotics excrete metabolites of these antibiotics in sewage water which enters water treatment plants and causes development of resistant strains.[11] As per study conducted in Nebraska State of America, anti-microbial resistant genes were found in streptococci and Gram-negative bacilli in surface water contaminated with human and animal faeces.[12] In Germany, Voigt *et al* confirmed the presence of MDR bacteria not only in sewage water but also in drinking water. In a recent study, waste-water treatment plants

and drinking water samples were investigated for presence of major Gram-positive and Gram-negative bacteria which may had acquired resistant genes through gene transferring mechanisms. The report revealed that these water sources are contaminated with resistant *Enterococci*, *Staphylococci*, *Enterobacteriaceae* etc. By using molecular biological tool like PCR, it was noted that these bacteria had acquired resistant genes against vancomycin, methicillin and other β-lactam antibiotics.[13]

Pharmaceutical industrial wastes containing antibiotics traces enter freshwater ecosystem from where water is used for community water supplies, putting the public at major health risk.[14] The aim of our study was to determine the frequency of antibiotic resistant genes in *E. coli*, i.e., *sul1*, *dfrA1*, *tetA*, *tetB* and *ermB* genes against major antibiotics groups including sulfonamides, trimethoprim, tetracyclines and erythromycin, in wastewater coming from Industrial Estate Hayatabad Peshawar.

MATERIAL AND METHODS

In this comparative study, water samples taken from industrial wastes were collected; treated water through Waste-Water Treatment Plants (WWTPs) was excluded from the study. A total of 25 random study samples were taken from 5 different industrial waste-water points at a depth of 0.5 m, and tested at Institute of Basic Medical Sciences, Khyber Medical University (IBMS-KMU) Peshawar. Presence of resistant *E. coli* in samples was confirmed by applying biochemical tests, i.e., citrate utilization test, indole test, and triple sugar iron (TSI) test. The *E. coli* was then cultured on MacConkey agar media and Gram staining was performed for identification of morphological features. *E. coli* colonies were recognized based on their morphological characteristics such as circular shape, pink colour, raised, entire margin, and smooth and shiny texture. Glycerol stock solution was prepared for preservation of these bacterial colonies.

DNA from cultured bacterial samples was extracted by the 'Boiling lysis' method.[15] Specific primers[16] were prepared, and presence of antibiotic resistant genes (ARGs) was detected with PCR. The amplification products were analyzed by loading 10 µL of PCR product on 1.5% agarose gel in Bio-Rad PowerPac® electrophoresis system along with 1 µL of 6× loading dye, and 1000 bp DNA ladder was used for size discrimination. The results were then visualized under Accuris E3000™ UV trans-illuminator. The frequencies and percentages of ARGs present in resistant *E. coli* strains were tabulated and calculated on Microsoft Excel 2016.

RESULTS

The citrate utilization test was negative, i.e., the colour of the media remained green which showed presence of *E. coli* in the medium. A positive indole test was observed by finding red colour ring on the upper layer of media which indicated the presence of *E. coli*. The results of triple sugar iron test (TSI) also showed positive results, i.e., appearance of yellow slant in growth media. For presence of ARGs in isolated *E. coli* isolates, specific primers were designed for each type of resistant gene. There was a high mean percentage of *sul1* gene in *E. coli* strains (84%) in all wastewater samples obtained from effluents of selected pharmaceutical industries. Mean percentage of *dfrA1*, *tetA* and *tetB* resistant genes was found to be 80%, while mean percentage of *ermB* resistant genes was 72% in our study samples. The frequencies of different ARGS in our study samples are shown in Table-1.

The results were then visualized under ultraviolet (UV) trans-illuminator (Figure-1).

Table-1: Frequency of antibiotic-resistant genes in study samples

Sampling Site	*sul1*		*dfrA1*		*tetA*		*tetB*		*ermB*	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Pharmaceuticals A	4	80	5	100	4	80	4	80	5	100
Pharmaceuticals B	3	60	4	80	5	100	4	80	3	60
Pharmaceuticals C	5	100	5	100	4	80	4	80	3	60
Pharmaceuticals D	4	80	4	80	4	80	4	80	3	60
Pharmaceuticals E	5	100	2	40	3	60	4	80	4	80
Mean Percentage	**84**		**80**		**80**		**80**		**72**	

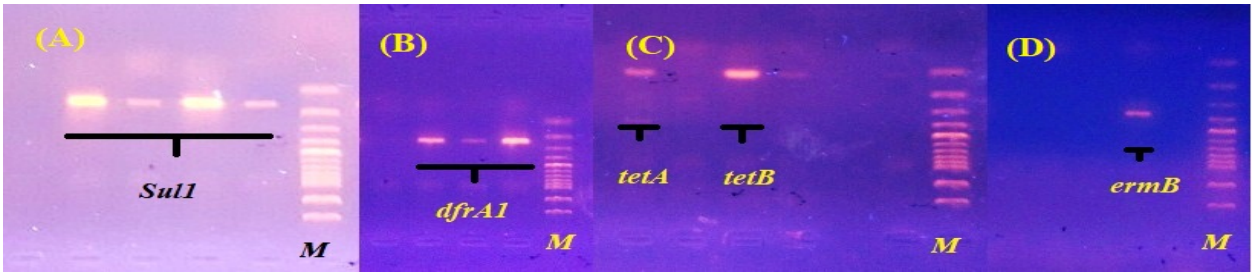

Figure-1: Antimicrobial Resistant Genes Amplification with 1000 bp DNA ladder
Left to Right: (A): *Sul1*, (B): *dfrA1*, (C): *tetA* & *tetB*, (D): *ermB*

DISCUSSION

Bacteria can acquire resistant genes sexually through the transfer of genetic material from one resistant bacterium to another non-resistant bacterium, i.e., conjugation, transduction and transformation mechanisms. This is known as 'horizontal evolution'.[17] Some bacteria transfer their genetically virulent genes to another species through bacteriophage viruses, which are responsible for horizontal gene transfer (HGT) from one bacterium to another.[18] Mobile genetic elements such as plasmids, transposons and gene cassettes, are also responsible for development of antibiotic resistance in bacteria.[19] Another mechanism by which bacteria can produce resistance against antibiotics is by the ways of efflux pumps, i.e., the pumping proteins that continuously kicks-out the antimicrobial agents out of the bacterial cell. Efflux pump is the major mechanism of antimicrobial resistance in *E. coli*, *Klebsiella pneumoniae*, *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and other pathogenic bacterial species.[20]

Several classes of antibiotics are used for treatment of mild to severe infections such as penicillin, cephalosporins, macrolides, tetracyclines and aminoglycosides etc. Recently, synthetic antibiotics like carbapenems had become the drug of choice for the treatment of severe infections caused by *Enterobacteriaceae*.[21] Over the last 50 years, antibiotic prescription by healthcare professionals for treatment or prophylaxis against infections has increased.[22] Similarly, an increase in demand for animal protein in developing countries leads to the un-opposed use of antimicrobial agents in agriculture and fish farming business for better production. The antibiotic residues in these livestock products ultimately lead to antimicrobial resistance in bacteria.[23,24] To meet the demand, the production graph of pharmaceutical industries has also been raised. The industrial waste of such pharmaceuticals is mostly expelled through water in the drainage system.[25] Due to inefficient water treatment, the wastewater of these pharmaceutical plants is more likely to contaminate the freshwater reservoirs with antibiotics residues.[26] In developed countries, effective pharmaceutical wastewater treatment has reduced the risk of onset of antimicrobial resistance. However, the effective biological treatment systems themselves serve as an ideal place for growth of different microbes and even a low concentration of antibiotic residues may result in development of antimicrobial-resistant genes (ARGs) in bacteria on prolong exposure.[27] Guo *et al* found high frequency of ARGs in wastewater samples taken from different pharmaceutical wastewater treatment plants.[28]

Antibiotics resistance is now an emerging problem in Pakistan. Many studies performed at different areas of the country revealed resistance to antibiotics commonly prescribed for the treatment of infectious diseases. Self-medication and lack of knowledge about the use of antibiotics has lead to development of isolated resistant strains of *Streptococcus pneumoniae* against co-trimoxazole, amoxicillin and chloramphenicol in Pakistan.[29] The high frequency of antimicrobial-resistant genes in the microbiota of major water reservoirs of Pakistan like rivers, canal systems and also wastewater from pharmaceutical plants is a growing health concern. This includes ARGs against antibiotics like penicillins, tetracyclines, sulfonamides, macrolides, and fluoroquinolones.[30] The main reason for it is that the water treatment plants are not a priority of these industries. There are no regulations for levels of antimicrobials residues in wastewater effluents from pharmaceutical industries. This is because of a lack of data on minimum concentrations of antibiotics which may cause development of ARGs in microbes. Recently, some limits for antibiotic residues in pharmaceutical effluents has been proposed in Europe which requires effective implementation by drug regulatory authorities in developing countries.[31] A recent study conducted in Vietnam revealed that the pharmaceutical discharged water contains high concentrations of antibiotic traces including fluoroquinolones.[32] Antimicrobial resistance is a growing concern all around the world especially in developing countries due to lack of appropriate health control system. Sufficient measures are required to be implemented by the government for control of production and use of antibiotics in healthcare facilities.

In our study, a total of 25 water samples were taken from 5 pharmaceutical manufacturing industries for the presence of ARGs in *E. coli*. Our results showed that different samples of wastewater effluents of 5 pharmaceutical industries contain resistant strains of *E. coli* against antibacterials such as sulfonamides, trimethoprim, tetracyclines and macrolides.

CONCLUSION

The effluents of pharmaceutical industries situated in Peshawar are a source of producing resistant strains of *E. coli.* A high percentage of *sul1* resistant gene followed by *dfrA1*, *tetA* and *tetB* resistant genes, while a relatively low percentage of *ermB* resistant gene was found in their wastewater effluents.

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