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ORIGINAL ARTICLE

Analysis of Antibiotic Resistant Gene from Microbial Isolates of Chlorinated Water

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ABSTRACT

Background: Chlorination is commonly used to disinfect water as it efficiently kills the microbe by destroying its membrane enzyme. Although some gram positive bacteria that are antibiotic resistant also show resistance against chlorine. This resistance may be due to some unique protein or maybe confer by the antibiotic resistant genes.
Aim: To analyze the relationship between antibiotic and chlorine resistance to verify the role of antibiotic resistant genes in chlorine resistance.
Methodology: VAN-A and TET-O genes were amplified from a chlorinated water sample and computation analysis was carried out to analyze these proteins. Docking was performed to quantify binding affinity between resistant genes and hypochlorite ligand.
Results: Low binding energy confirmed the incompetency of chlorine and antibiotics against resistant genes. Expression analysis of PET-28 GFP recombinant plasmid under antibiotic and chlorine stress indicated successful expression by J express.
Conclusion: Computational study proves that antibiotic resistant gene has a role in conferring resistance against chlorine.
Keywords: Antibiotic resistance, chlorine resistance, molecular modeling, recombinant plasmid, hypochlorite

INTRODUCTION

Safe water is essential for the good health and wellbeing of society, according to WHO report, about 1.1 billion people drink unsafe water[1]. Due to the lack of clean, pure water and basic sanitation facilities, annually 3.7% of the deaths occur global. Despite of the germicidal activity of chlorine, some microorganisms remain alive in water as several studies demonstrate various microbes from water circulation framework where chlorine particles remain free. These chlorine resistant microbes serve creates severe medicalcomplications [2]. Olson and Ridgway reveal that microorganisms present in chlorinated water are part of residues, and hence were not degraded by chlorine[3]. Gram positive bacteria are more resistant than gram negative bacteria as the isolation of gram positive bacteria from chlorinated water in the presence of chloramphenicol indicate aggregation of bacteria and production of unique protein.

In silico analysis is an advance method to be used for various purposes because it is cost effective and labor saving analysis method for research. So far, dry lab analysis was not performed on chlorine resistant bacteria.Therefore, the present study focuses on the *in silico* computational analysis followed by*in vitro* analysis. Consequently, the chlorinated water was subjected to authentic processesforthe validation and identification of bacterial strain. Polymerase Chain Reaction (PCR)specifies the chlorine resistant bacteria to be the antibiotic resistant because these are amplified by antibiotic resistant genes and 16SRNA. To identify the exact sequence of bacteria.Molecular docking and simulations were conducted to evaluate the interaction and resistant pattern of microbe with chlorine. In Pakistan, waterborne diseases accounts for 80% of all the microbial infections including hepatitis, intestinal worm, typhoid cholera, giardiasis, diarrhea, dysentery, and cryptosporidium leading to 33% of deaths[4]. The presence of resistance microbes in chlorinated or disinfected water increases the potential health risk of using that water for drinking and domestic purposes. Therefore present study highlighted the inadequate application of water disinfectant methods and treatment options. Moreover, in this study, the bacterial resistanceto chlorine was evaluated by*in silico* and *in vitro* analysis procedures.

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MATERIAL AND METHODS

Sample collection and identification of resistant bacteria: The samples of chlorinated water was collected from laboratory using aseptic techniques in a sterile tubes and stored at 4°C. To verify the presence of resistant bacteria water sample with various concentration of chlorine (100,000, 10,000, 5000, 1000, 50, 10ppm) was incubated at 37°C for 24 hrs on LB agar plates. Gram staining was performed on the chlorinated water sample. The polymerase chain reaction was carried out for 16sRNA using the following forward (5´- AGAGTTTGATCMTGGCTCAG-3´) and reverse (5´-CGGTTACCTTGTTACGACTT-3´) primers to visualize and obtain gel electrophoresis bands on 2% agarose gel.

Amplification and sequencing of antibiotic resistant genes; VAN-A & TET-O: Isolated DNA was subjected to PCR to amplify VAN-A and TET-O for antibiotic resistant genes VAN-A was amplified at 53°C by using forward 5´-GGGAAAACGACAATTGC-3´ and reverse 5´-GTACAATGCGGCCGTTA-3´ primers. TET-O was amplified at 56°C by using these 5´-ACGGARAGTTTATTGTATACC-3´ and 5´-TGGCGTATCTATAATGTTGAC-3´ sequences respectively. Bacterial isolates were sequenced from MACROGEN (Korea).

Insilico **physiochemical, secondary structure and gene ontology prediction:** Physiochemical chemical properties was analyzed using an online tool, EMBOSS Pepstats(https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/). PSIPRED v3.3 and CFSSP was employed to predict the secondary structure of VAN-A and TET-O and protein domains were speculated by Inter Pro Scan (https://www.ebi.ac.uk/interpro/search/sequence-search). An online bioinformatics tool, GO tool (http://www.geneontology.org) was used to develop structurally controlled ontologies based on their biological processes, cellular components and molecular functions.

Molecular modeling and simulation of resistant proteins: 3D structure of Van-A and TET-O was generated and predicted by an online server I-TASSAR (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). Antibiotic resistant protein models were selected based on C-score. For molecular docking simulations, specific 3D structure ligand hypochlorite was retrieved from largest data bank of chemical information, PubChem (https://pubchem.ncbi.nlm.nih.gov)and visualized molecular system PyMOL (https://pymol.org)as displayed in Figure 1. For analysis of molecular docking and simulation interactions another tool named

as Auto Dock Vina (https://vina.scripps.edu) was used. The recombinant Pet28-GFP plasmid was produced by inserting antibiotic resistant genes (VAN-A & TET-O) along BamH1 and Xhol1 restriction sites to check the expression of respective gene under antibiotic stress.

RESULTS

Bacterial screening indicated that 100,000 ppm concentration of chlorine is considered effective to control all types of resistant bacteria. Polymerase chain reaction (PCR) for VAN-A gives clear band of 163bp.However, PCR for TET-O gives band of 171bp on 2% agarosegel are detailed described in Figure 2. The sequenced sample of TET-O indicates the highest similarity with *Actinobacillus, Streptococcus and Bifidobacterium*species and that of VAN-A illustrate highest similarity with *Enterococcus, Staphylococcus and Enterococcus*species according to BLAST similarity and identity index. Secondary structure analysis estimated that antibiotic resistant VAN-A contains 75.2% helix, 60.2% of sheets and 18.2% of turn in the protein whereas TET-O gene comprised of 75.4%, 53.1% and 12.4% of helix sheets and turn, respectively. Inter proscan determine several domains, sites and function of these resistant protein including P-loop containing nucleotide triphosphate hydrolase, translational protein beta barrel domain superfamily, EF-G domain III, ribosomal protein,5-domain 2-type fold, small GTP binding domain, transcriptional factor-GTP binding domain and putative GEF interaction site, GTP/Mg2+ binding site, as function. Moreover, physiochemical properties and Gene ontology analysis to predicts molecular weight, iso electric point, net charge, cellular, molecular and biological function is summarized in Table 1. The predicted structure of VAN-A showed identity with the pseudomonas 145 cellulose alpha-D glucuronidase (1GQLA) with a TM score of 0.41 and the structure of TET-O showed identity with E.coli 70s ribosome with antibiotic TetM (5Kcs1w) with a TM score of 0.985.

Auto dock vina docking analysis exhibit several binding energies between hypochlorite and antibiotic resistant proteins that was further interpreted by 3D simulation on PYMOL as shown in Figure 3. Low binding affinity among the resistant gene and hypochlorite as -2.0 kcal/mol for TET-O and VAN-A, -1.7 kcal/mol for 1T1F domain of TET-O, -2.5 kcal/mol for 2HF8 and -2.3 kcal/mol for 4MYT thus confirmed the low effect of chlorine or antibiotics on these genes as shown in Table 2.

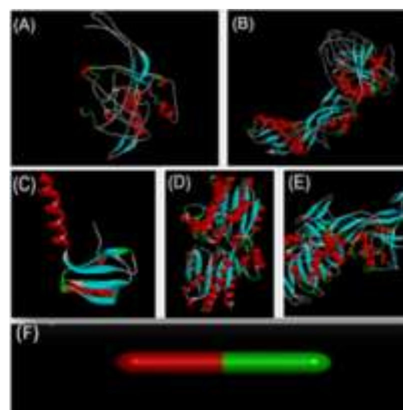

Figure 1. 3D structure of resistant proteins and ligand (A) VAN-A Staphylococcus (B) TET-O Staphylococcus (C) 1tif: domain of TET-O (D) 2HF8: domain of TET-O (E) 4MYT: domain of TET-O (F) Ligand; Hypochlorite (Pub Chem ID; 61739)

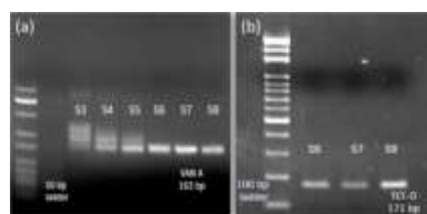

Figure 2. (a) PCR amplification of VAN-A (b) PCR amplification of TET-O

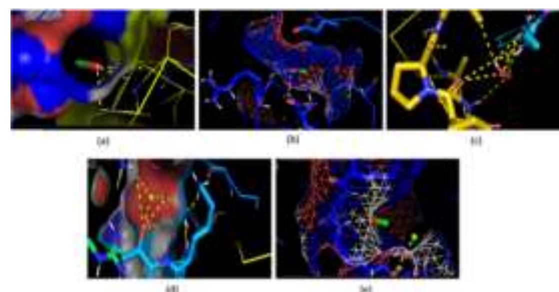
Figure 3. 3D ligand and protein docking complexes visualized on PYMOL (a) VAN-A with hypochlorite (b) TET-O with hypochlorite (c) 1TTIF with hypochlorite (d) 2HF8 with hypochlorite (e) 4MYT with hypochlorite

Table 1: In silicoanalysis

Table 1.*In silico*analysis	Physiochemical properties		Gene ontology			
	Protein Residue	274aa	Molecular function		Biochemical function	
	Weight	31356.3 g	GO term	Function	GO term	Function
	Net Charge	13.0	GO-0003924	GTPase activity	GO- 0006184	GTP catabolic process
	Isoelectric Point	9.8007	GO-0003746	Translational elongational factor	GO-0006414	Translational elongation
TET-O	Protein Residue	639aa	Molecular function		Biochemical function	
	Weight	72353.23	GO term	Function	GO term	Function
	Net Charge	-9.0	GO-0043167	Ion binding	GO-0055114	Oxidation reduction
	Isoelectric Point	5.4403			GO-0007155	Cell adhesion

Table 2. Resistant proteins binding affinity with ligand hypochlorite

Protein- Ligand Complex	Affinity
VAN-A- Hypochlorite	-2.0 kcal/mol
TET-O- Hypochlorite	-2.0 kcal/mol
1TIF- Hypochlorite	-1.7 kcal/mol
2HF8- Hypochlorite	-2.5 kcal/mol
4MYT-Hypochlorite	-2.3 kcal/mol

DISCUSSION

Various factors contribute to the introduction or presence of bacteria into the water and the main reason is the improper sanitation. Hence proper disinfection is required for the removal of these pathogenic microbes[5]. The one of the advance techniques of disinfection is the chlorination of water in various form either chlorine gas or sodium hypochlorite or calcium hypochlorite or hypochlorous acid[6]. These chlorine molecules eradicate the bacteria by inhibiting the activity of membrane enzyme and ultimately destroying the microbial cell.

Despite of the fact thatnumber of resistantbacterial species may survive even in the presence of disinfectant with chlorine. This may be due to several reasons, as there are various ways for identification of resistance mechanismhowever there is lack of evidence for detail phases involved. Furthermore, all the bacteria

that were isolated from the chlorinated water were surprisingly, found to be antibiotic resistant[7]. Bacteria that survive in water may have innate ability of resistance as the spore forming bacteria are mostly resistant and gram-negative.Moreover, resistant bacteria are considered to be less sensitive than the gram positive-bacteria which means that gram negative bacteria could survive in chlorinated water for long[5].

This study was then carried out simultaneously computational and molecular lab for detail validations and verifications. The physicochemical properties of resistant protein were determined by bioinformatic tool named as Emboss Pepstats that predict its weight, isoelectric point etc. secondary structure of protein was generated by PSIPRED tool. The secondary structure consists of four component including helix, sheets, turns and coils. Protein has many domains that are involved in various function as predicted by Interpro scan (https://www.ebi.ac.uk/interpro/search/sequence/) .and the molecular and biological function of protein was predicted by GO tools. The 3D structure of protein was generated by I-TASSER (https://zhanggroup.org/I-TASSER) and was allowed to interact with the hypochlorous acid/ hypochlorite structure for docking analysis. The affinity of protein with ligand was recorded around -2.0, indicating that the proteins are resistant to ligand.

In this study for the conformation of the role of antibiotic resistant genes in chlorine resistance, vector was also constructed. The Snap gene software (https://www.snapgene.com/) was used to insert the VAN-A and TET-O genes in Pet28-GFP plasmid. This vector is a fluorescent plasmid and will produce fluorescence upon expression. After insertion the vector was subjected to antibiotic and HOCl stress and expression was observed as proportional to the resistance ability of protein[8].

Most water borne diseases are caused by resistant microbe that are present in contaminated water including *Enterobacter* (64%), followed by *Salmonella, E.coli*, *Cyclosporincoetaneities, Giardia lamblia* and *Clostrium* that owing to their resistant ability, remain survived in disinfected water[9]. This study further elaborates the presence of pathogens in chlorinated water thus deploying the need for better sanitation practices for improved community health and health quality.

CONCLUSION

In conclusion the antibiotic resistant genes plays a vital role in conferring the resistance against chlorine in water. As the antibiotic resistant genes were found to be present in all chlorine resistant microbial isolates and the expression of recombinant plasmid also prove that antibiotic resistance co-select with t chlorine resistance. However, the exact mechanism of resistance is ambiguous hence further future advance study should be conducted to explore the underlying mechanism of resistance against chlorine in water.

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