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Microbial Profile and Heavy Metals Resistanceamong Municipal Wastewater (Al-Yohedia Stream) in Hilla City, Iraq

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Abstract : Municipal Wastewater mainly composed of chemical pollutants (like heavy metals) and biological pollutant (like microbes). Municipal waste water samples were collected from four sites along Al-Yohedia Stream in Hilla City, Iraq and the samples were subjected to study the heavy metals concentration, Microbial profile, Antibiogram and presence of heavy metals efflux pump (CusCFBA) among Gram-negative isolates. The results revealed occurrence of high concentration of heavy metals includes (from high to low): Nickel> Manganese> Iron> Cadmium> Copper >Lead. The results also display the presence of many pathogenic bacteria and Candida spp. in all four sites includes: Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis, Vibrio cholerae and Vibrio parahemolyticus. The candida spp. includes: Candida albicans, Candida krusei and Candida glabrata. Among Gram-negative isolates, high resistance (91.66% and 83.33%) were recorded for Amoxicillin-clavulanic acid and cefotaxime respectively while low level of resistance were showed for the rest. Resistance to clarithromycin were noted to all S. aureus isolates and vancomycin resistance E. faecalis were also documented. Full resistance to ketoconazole and miconazole while resistance to itraconazole and fluconazolewere (90.90% and 81.81%) respectively. The presence of CusCFBA efflux pump among enterobacteria were verified via presence of outer membrane protein gene (*cusC*) among three isolates of E. coli and one isolate of K. pneumonia. This study conclude high concentration of most of heavy metals among waste water and emergence multidrug resistance and heavy metals resistance bacteria and Candida spp. and this resistance may attributed to presence of heavy metals and drug efflux pump among those microbes especially CusCFBA pump among Gram-negative isolates.
Keywords : Heavy Metals, Microbes, CusCFBA.

Introduction:

Municipal wastewater is mixture of domestic wastewater, industrial wastewater, storm water, and groundwater seepage. Domestic wastewater consists of effluent discharges from households, institutions, commercial and industrial buildings[1]. Municipal Wastewater can be characterized by its main contaminants which may have negative impacts on the aqueous environment. It mainly composed of solids like sand, grit, organic matter, micro-pollutant ,total dissolved solids like salts, microorganisms, biodegradable organic materials, other organic materials like detergents, pesticides, fat, oil and grease, cyanide, nutrients like nitrogen, phosphorus, heavy metals like Hg, Pb, Cd, Cr, Cu, Ni, other inorganic materials like acids, hydrogen sulphide and radioactive materials[2-4].

Pathogens commonly found in wastewater and of concern to wastewater personnel include viruses, bacteria, fungi, protozoans, and helminthes. Allergens, endotoxins, and exotoxins are also found in wastewater and represent a concern to wastewater personnel .The commonly pathogens existed in wastewater include: Vibrio cholerae, Escherichia coli, Klebsiella spp., Salmonella spp., Shigella spp., Hepatitis viruses, Norwalk virus, Candida, Cryptosporidium and Schistomsoma spp. The main entrance of these pathogens into water is also through fecal contamination[5,6]. Al-Yohedia stream pass through people inhabited regions (Al-Karama, Al-Gamiyah and Al-Eskan quarters) and many pollutants, used gasoline engine oil, houses sewage water drain in it. Heavy metals can cause DNA damage to bacterial genome and the resistance to this metals regards a virulence factor for pathogen in which it present[7,8].The problem is sewage water of Al-Yohedia stream can reach to neighboring houses (located along the two sides) via cracks in the tap water networks. Also it can be reach to the vegetative farms at the end of this stream (all of farms uses this water for irrigation). The comprehensive study for this stream not done yet and the current work aim to study the microbial profile and heavy metal resistance of this stream.

Materials and Methods:

Sample Collections and Heavy Metals Measurement:

Ten liters of municipal sewage samples were collected from four sites, after samples processing with filtration and concentration by acids, Flame Atomic absorption-spectrophotometer model7000A (Shimadzu /Japan) was used to determine the heavy metals concentrations (mg/L)[9].

Total Plate Count:

All samples mixed well and 1 ml of waste water sample transferred to 9 ml of normal saline (diluent) and six serial dilution (10-1, 10-2, 10-3, 10-4, 10-5, 10-6) were prepared. A 10 µl from the last dilution (sixth dilution) and spreading them on nutrient agar plate and incubate the plates upside down in 37 °C for 24 h. A triplicate were performed to each sample to increase the accuracy and only plate that have 30-300 colonies will be chosen for count[10]. The exact number of colonies per original sample will calculated according to the following equation:

CFU/ml = no. of colonies x inverse of the dilution x 100.

Microbial Isolations and Identification:

Immediately after sample collection and along with total plate count the samples inoculated on blood agar, UTI chromogenic agar, Pseudomonas chromogenic agar, Vibrio chromogenic agar and Candida chromogenic agar to investigate the presence of common pathogens among wastewater samples. The sample mixed well and from each sample a triplicate of 100ml will filterated on 0.45 µm cellulose nitrate membranes (Sartorius/Germany) using Sartorius filtration system and then the filter content resuspended in sterile normal saline and cultured on the above mentioned agar, incubated at 37 °C for 48 h. The diagnosis of grown bacteria and candida will be according to the chromogenic guide provided with medium[11]. E. coli give a faint pink colonies while K. pneumoniae give dark blue colonies on UTI chromogenic agar. Further differentiation via their growth on EMB agar (green metallic shine for E. coli while mucoid, deep purple colonies for K. pneumoniae). S. aureus give white creamy colonies while E. faecalis give light blue to green colonies on UTI chromogenic agar and then confirmed by catalase assay and growth on mannitol salt agar. In addition E. faecalis were confirmed by PCR. Oxidase positive colonies of P. aeruginosa appear as blue to purple colonies on selective Pseudomonas chromogenic agar. Vibrio cholerae appear as pink-rose colonies while Vibrio parahaemolyticus appear as blue-green on selective vibrio chromogenic agar[12]. Selective candida chromogenic agar were used for isolation and differentiation between Candida spp.. C. albicans give green-blue colonies, C. krusei give pink-orange colonies and C. glabrata give light white colonies[13].

Antimicrobial susceptibility test by agar disk method:

The in vitro antibiotic and antifungal susceptibility were determined via disk diffusion method according to Clinical and Laboratory Standards Institute instructions (CLSI, 2016)[14]. Activation of isolates were performed using nutrient broth for 18 h at 37°C and the growth was adjusted to 0.5 McFarland's standard (108 CFU/mL) and then spread on Muller Hinton agar (MHA) with a sterile cotton swab. Antibiotic disks were placed onto MHA,

gently pressed down to ensure complete contact with the agar inoculated with bacteria and then incubated for 24 h at 37°C and then inhibition zone diameter in millimeters (mm) was recorded. Interpretation of results as a sensitive or resist were achieved according to CLSI, 2016.

Bacterial DNA Extraction:

Favor PrepTM Genomic DNA Mini Kit was used to extract genomic DNA from bacterial isolates according to the manufacturer's protocol. For Gram-positive isolates additional step (using lysozyme+TE buffer) were performed as preparation for DNA extraction.

Primer Pairs Preparation:

All primer pairs used in this study were dissolved using TE Buffer, 1X (pH 8.0) composed of 10mM Tris-HCl containing 1mM EDTA-Na2. Firstly the primer stock tube prepared and then the working solution would prepared from primer stock tube. According to the instruction provided by primer manufacturer (Bioneer / Korea) the TE buffer were added to get 100 picomole/microliter concentration of primer stock solution. The working solution prepared from stock by dilution with TE buffer to get 10 picomole/microliter[15].

Polymerase Chain Reaction (PCR):

Conventional PCR were used to amplify the target DNA using specific primer pairs. It include three consecutive steps that repeated for specific number of cycles to get PCR product (amplicon) which can be finally visualized after agarose gel electrophoresis. The primer sequence, PCR product size and thermal cycling conditions mentioned in the table (1)

Table (1): Primer Sequence and Thermal Cycler Condition.

Gene	Primer sequence ('5-3')	Step	Condition	Product	References
cusC	F:CGCCTTTAAAGAAGTGGCAG R:CTGACGGGCATAATTCAGGT	Initial Denaturation	95 ºC 4 min. 1 cycle	217 bp	16
		Denaturation	94 ºC 30 sec. 30 cycles		
		Annealing	59 ºC 30 sec. 30 cycles		
		Extension	72 ºC 30 sec. 30 cycles		
		Final Extension	72 ºC 5 min. 1 cycles		

Statistical Analysis:

SPSS programs used for least significance differences (LSD $\leq$ 0.05), Analysis of variance test (ANOVA) between sites and different Studies parameters. Also Canoco analysis (Canonical correspondence analysis) used with original Version 4.0.

Biosafety and Hazard Material Disposing:

Biosafety aspects followed during the work include disposing of all swabs, petri dishes and all contaminated supplies by autoclaving and then incineration. All benches cleaned with alcohol before and after the work. Simply safe were used instead of ethidium bromide.

Results and Discussion:

Dissolved Heavy Metals:

Dissolved heavy metals showed variations among study sites. Most of the concentrations of measured metals fluctuated during study period. Pb concentration ranged (0.8000-3.0000 mg /l) were the lowest value in Site 4 while highest value in Site 2 and Site 3, Cu ranged (4.0000- 6.0000 mg /l) were lowest value in Site 3 while highest value in Site 1. Cd ranged (12.0000- 16.0000 mg /l) where the lowest value in Site 1 and Site 2 while the highest value in Site 3. Fe ranged (30.6000- 55.0000 mg /l) were the lowest value in Site 1 while the highest value in S2. Mn ranged (100.1000- 116.6667 mg /l) where the lowest value in Site 2 whiles the highest value in Site 1. Ni ranged (119.0000– 120.7000 mg /l) where the lowest value in Site 2 and Site 3while the highest value in Site 1. Some significant differences according to statistical analysis and all previously mentioned concentrations of heavy metals have been explained in table (2) and figure (1).

Statistical Analysis showed that Cd has not significance differences between Site 1 and Site 2 and between Site 3 and Site 4 while has significance differences between Site 1 andSite 3 and Site1 and Site 4 and between Site 2 and Site 3 and Site 2 andSite 4. Fe do not has significance differences between Site 1 andSite 3 while has significance differences between Site 1 andSite 2 and Site 1 and Site 4 and Site 2 andSite 4 and between Site 3 and Site 2 and Site 3 and Site 4 . Cu ,Pb ,Ni do not have significance differences in all sites while Mn has significance differences in all sites. All measured metals followed this trend (Ni>Mn>Fe>Cd>Cu>Pb).

Table (2): Variation of dissolved heavy metals of municipal wastewater in 4 sites.

Dissolved (mg /l)	Site 1	Site 2	Site 3	Site 4
Cu	6 a ± 0.01	5 **a** ± 2	4 **a** ± 1	**5** a ± **0.01**
Mn	116.6667 d ± 2.08167	100.1 **a** ± 0.1	110.2333 c ± 0.25166	**106 b ± 2**
Fe	30.6 **a** ± 0.4	55 c ± 3	32 a ± 0.01	**44.3333 b ± 1.52753**
Cd	12 a ± 1	12 a ± 0.01	16 b ± 2	**15 b ± 1**
Pb	2.7667 **a** ± 0.66583	3 **a** ± 2	3 **a** ± 2	**0.8 a ± 0.01**
Ni	120.7 a ± 0.1	119 **a** ± 1	119 **a** ± 0.01	**120** a ± **2**

p<0.05

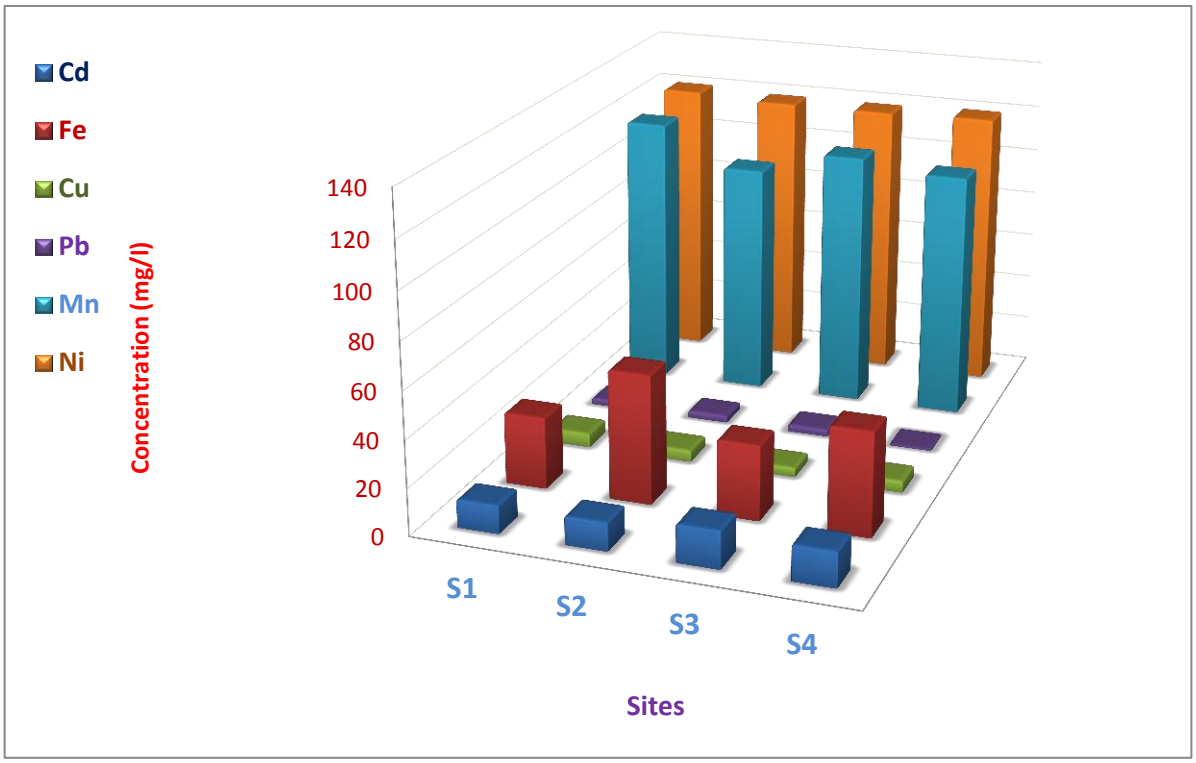

Figure (1): Variations in concentration of dissolved Cadmium (Cd), Iron (Fe), Copper (Cu), Lead (Pb), Manganese (Mn) and Nickel (Ni) among Four Sites.

Total Plate Count:

The results of total plate count reveal high microbial content of wastewater in all sites and there are non-significance differences among sites (table 3).

Table (3): Total Plate Count of bacteria among Four Sites.

Sites	Site 1	Site 2	Site 3	Site 4
	Mean x10^{10}±SD			
CFU\ml	2.25 ±1 [a]	1.88±0.11 [a]	1.98±0.86 [a]	1.75±0.2 [a]

Although the distance among four sites were long (about 1 kilometer between each two sites) and the microbial content must be decreased due to low flow of the Al-Yohedia Stream, but the microbial content still high and this can be explained as a result to continuous suppling of microbes via different types of contaminated waste water (municipal, nurse-house, Butcher-house, medical college and animal-house waste water) located at the side of this stream. Biomedical or health care waste is Biomedical wastes are extremely hazardous type of waste and if not treat properly, can cause serious health and environment problems[17-20].

The wastewater sample from site 4 (Al-Askari quarter (N32 ^{0} 25.827 E044 ^{0}24.394) have a Mean±SD of microbial content equal to 1.75 x 10^{10} ±0.2and we found that it be used for irrigation and this is a big problem. Blumenthal et al., (2000)[21] stated that the treated wastewater microbial content must be zero or range 10^3-10^5 CFU/100ml (10-1000 CFU/ml) to be accepted for irrigation.

Pathogenic Bacteria and Yeast Isolates:

Results of bacterial and yeast isolation revealed that that Gram-positive, Gram-negative and *Candida* spp. were recovered all four sites. The isolates include E. coli, K. pneumoniae, P. aeruginosa, V. cholerae, V. parahaemolyticus, S. aureus, E. faecalis, C. albicans, C. krusei and C. *glabrata* (table 4). Most of the isolated bacteria and yeast have clinical importance and push a risk for human health. Our results in agreement with Yang

et al., (2009)[22] who found that the clinical isolate of *E. coli*, *K. pneumoniae*, *S. aureus* and *P. aeruginosa* were common among sewage water. Results of the current study concern presence of Candida spp. were supported by Filipkowska et al., (2008)[23], Biedunkiewicz and Ozimek (2009)[24] and Korzeniewska et al., (2009)[25] whom recovered the different species of Candida from municipal wastewater.

Table (4): Distribution of isolated Pathogen according to Sites.

Pathogen	Site1	Site2	Site3	Site4
Escherichia coli	+	+	+	+
Klebsiella pneumoniae	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+
Vibrio cholerae	+	+	+	+
Vibrio parahaemolyticus	+	+	+	-
Staphylococcus aureus	+	+	+	+
Enterococcus faecalis	+	+	+	+
Candida albicans	+	+	+	+
Candida krusei	+	+	+	-
Candida glabrata	+	+	+	+

The availability of potentially pathogenic fungi of genus Candida, particularly Candida albicans, in municipal wastewater considered as marker for their direct source from the human body, mainly the preliminary system[25-27]. The transfer of waste water stream among populated regions and using of the waste water in irrigation pushing hazard for human health and possibility of transmission of medically important bacteria and yeast to the people living across this stream[28].

Antibiotic Susceptibility forGram-negative Bacteria:

The results revealed high resistance of Gram-negative bacterial isolates to β-lactams (91.66% for amoxicillin/clavulanic acid) and cephalosporins (83.33% for cefotaxime 66.66% for Ceftazidime). Resistance to aztreonam was 50% while for amikacin (41.66%). All isolate were sensitive to ciprofloxacin while very low resistance was expressed to gentamicin (8.33%) and to imipenem and trimethoprim-sulfamethoxazole (16.66% for both) (table 5). Our records were in accordance with those gathered by many studies on antibiotics resistance among waste water Gram-negative pathogens[28,29].

Table (5): Antibiotic susceptibility among Gram-negative bacterial isolates.

Isolate	Antibiotics								
	AMC	**CTX**	**CAZ**	**ATM**	**IPM**	**AK**	**CN**	**CIP**	**STX**
E1	R	R	S	S	S	S	S	S	S
E2	S	R	R	R	S	S	S	S	S
E3	R	R	R	R	S	S	S	S	S
E4	R	R	S	S	S	S	S	S	S
K1	R	R	R	R	S	S	S	S	S
K2	R	S	S	S	R	R	S	S	S
K3	R	S	R	R	R	S	R	S	S
K4	R	R	R	S	S	R	S	S	S
P1	R	R	R	R	S	R	S	S	S
P2	R	R	R	S	S	R	S	S	R
P3	R	R	R	R	S	S	S	S	S
P4	R	R	S	S	S	R	S	S	R
Resistance %	**91.66**	**83.33**	**66.66**	**50**	**16.66**	**41.66**	**8.33**	**0**	**16.66**

AMC= Amoxicillin/Clavulanic acid, CTX= Cefotaxime, CAZ= Ceftazidime, ATM= Aztreonam, IPM= Imipenem, AK=Amikacin, CN=Gentamicin, CIP= Ciprofloxacin, SXT= Trimethoprim-sulfamethoxazole

Developing of resistance to β-lactams and cephalosporins and aztreonam can be clarified as a result to carrying genes encodes for extended spectrum β-lactamases (ESBLs)like TEM-1, OXA-1, CTX-M and SHV. ESBLs genes located on bacterial chromosomes or may be exchanged among species and genus via transposable elements like plasmids. Production of extended-spectrum β-lactamases (ESBLs) is a significant resistance-mechanism that impedes the antimicrobial treatment of infections caused by *Enterobacteriaceae* and is a serious threat to the currently available antibiotic armory[30-34]. The resistance to Trimethoprim-sulfamethoxazole may be due to acquisition of dihydrofolatereductase (DHFR) and dihydropteroate synthase (DHPS) genes through mobile genetic elements such as plasmids, transposons, and class 1 integrons[35, 36].

The high concentrations of different types of antibiotics that reach waste water may be leads to emergence of antibiotics resistance among waste water bacteria, unabsorbed antibiotic residues are excreted in urine and feces, and ultimately transfer to wastewater via domestic sewer[37]. Classes of antibiotic residues that have frequently been detected in municipal effluents include *β*-lactam, macrolides, lincosamide, tetracyclines, sulphonamides, and fluoroquinolones[38].

Antibiotic Susceptibility for Gram-positive Bacteria:

Resistance among Gram-positive isolates was lower than those of Gram-negative. only two isolate of E. faecalis were shows resistance to vancomycin and may refer to vancomycin resistance enterococci (VRE). Our results in accordance with Talebi et al., (2008)[39]Araújo et al., (2010)[40] and Goldstein et al., (2013)[41] who recover vancomycin-resistant enterococci (VRE) at Iran, Portugal and U.S. wastewater treatment plants respectively that provide effluent for reuse. Resistance to vancomycin is due to the presence of operons that encode enzymes which modify the target of vancomycin. Resistance to clarithromycin and tetracycline were also recorder among S. aureus (table 6).

Our results agrred with Jiang et al., (2013)[42], Moges et al., (2014)[43] and Guo et al. (2014)[44] whom report the presence of resistance genes for tetracyclin and trimethoprim-sulfamethoxazole in drinking water treatment plants (DWTPs) and finished water and report 39 antibiotics resistance genes (ARGs) for tetracyclin, chloramphenicol and *β*–lactam in drinking water sources.

Table (6): Antibiotic susceptibility among Gram-positive Bacterial Isolates.

Isolate	Antibiotics									
	VAN	**AK**	**CN**	**CLR**	**TE**	**CIP**	**NOR**	**F**	**SXT**	**C**
Sa1	S	S	S	R	R	S	S	S	R	S
Sa2	S	S	S	R	R	R	R	S	R	S
Sa3	S	S	S	R	S	S	S	S	S	S
Sa4	S	R	S	R	S	S	S	S	S	S
EF1	S	NA	NA	NA	R	S	S	S	NA	S
EF2	R	NA	NA	NA	R	S	S	S	NA	S
EF3	R	NA	NA	NA	S	S	S	R	NA	S
EF4	S	NA	NA	NA	S	S	S	S	NA	S
Resistance %	25	25	0	100	50	25	25	25	50	0

VAN=Vancomycicn, AK=Amikacin, CN=Gentamicin, CLR=Clarethromycin, TE=Tetracyclin, CIP=Ciprofloxacin, NOR=Norfloxacin, F=Nitrofurantion, SXT= Trimethoprim-Sulfamethoxazole, C=Chloramphenicol.

The availability of outer membrane and LPS in G^+ bacteria and resistance or tolerance to high salt concentration in Gram positive bacteria may play explain the survive the wastewater pathogen in highly polluted wastewater especially with different types heavy metals and antibiotics[45].

The necessary to point that most of modern studies showed that heavy element resistant bacteria are also resistant to most of antibiotics and other toxic materials by mean of carrying plasmids and or transposons encoding genetically linked metal and antibiotic resistance. In addition to that, some researches detected some evidence that in wastewaters samples there is a high capability for horizontal gene transfer, mediated by plasmids

and facilitated by integrons[46,47]. Plasmids carrying resistance genes have been found in pathogenic bacteria of the genus Escherichia, Salmonella, Shigella, Klebsiella, Aeromonas, and Pseudomonas. These plasmids carry resistance to heavy metals such as nickel, cadmium, cobalt, silver, mercury, lead and zinc and drugs of different groups [Tetracyclines, quinolones, aminoglycosides, sulfonamides, β-lactams and chemotherapeutics. Similar to above, most results obtained from study on many clinical and environmental isolates reveal also that heavy metal and antibiotic resistance were often closely associated[48,49].

Antifungal Susceptibility for Candida spp.:

Resistance of Candida spp. to six antifungal were assessed and the results interpreted according to CLSI (2002). The isolates were fully resist to ketoconazole and miconazole while (90.90%) and (81.81%) of isolates showed resistance to itraconazole and fluconazole respectively. Lower resistance to econazole (58.33%) and for clotrimazole (27.27%) were also reported in the current study (table 7).

Table (7): Antifungal susceptibility among *Candida* spp. isolates.

Isolate	KCA	MCL	ECN	FLU	ITC	CLO
Cal1	R	R	R	R	R	S
Cal2	R	R	S	S	S	S
Cal3	R	R	S	S	R	S
Cal4	R	R	S	R	R	S
Ckr1	R	R	R	R	R	R
Ckr2	R	R	R	R	R	R
Ckr3	R	R	R	R	R	R
Cgl1	R	R	R	R	R	S
Cgl2	R	R	R	R	R	S
Cgl3	R	R	S	R	R	S
Cgl4	R	R	R	R	R	S
Resistance %	**100**	**100**	**58.33**	**81.81**	**90.90**	**27.27**

KCA= Ketoconazole, MCL= Miconazole, ECN= Econazole, FLU= Fluconazole, ITC= Itraconazole, CLO= Clotrimazole

The resistance to antifungal like azoles among candida spp. may attributed to *Candida* drug resistance (CDR) genes have been associated with azole resistance. At least 5 different CDR genes (*CDR1* to *CDR5*) have been described. *CDR1* is a transporter protein in *Candida* spp. [50,51].

Heavy Metals Efflux pump (CusCFBA):

In general the *S. aureus, E. faecalis and Candida spp*. can resist or tolerate high concentration of salts and heavy metals may be due to the structure of their wall. Enterobacteria can evolve a mechanisms to survive among high salt and heavy metals niches like waste water and the important one among them is efflux pump. The PCR results revealed the presence of *cusC* (encodes for outer membrane part protein of CusCFBA pump) in three isolate of *E. coli* and one isolate of *K. pneumoniae* (figure 2) . Our results were in accordance with Al-Dahmoshi et al., (2017)[16] who found *cusC* in 9/11 of uropathogenic E. coli (UPEC) isolates.

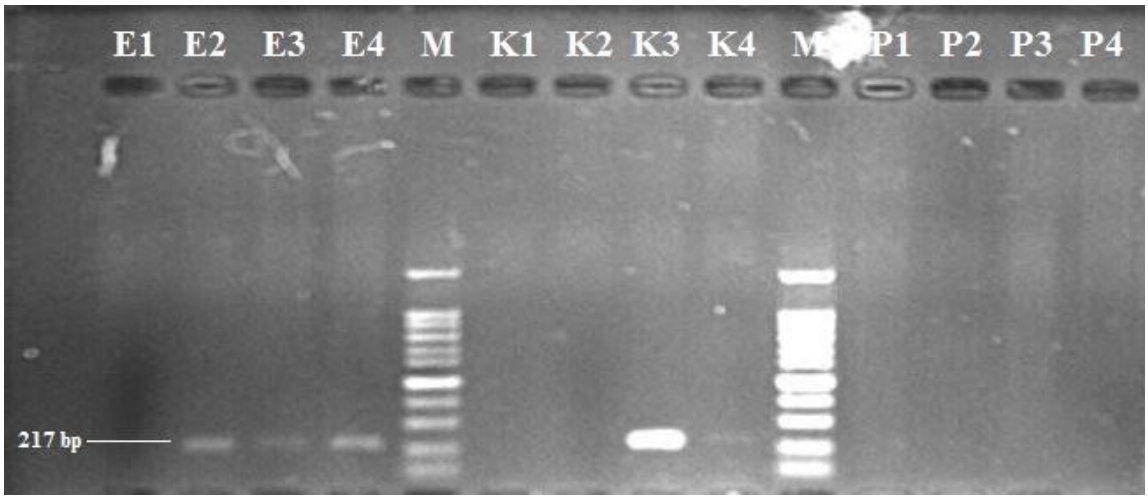

Figure (2): Agarose gel electrophoresis (1.5%) for *cusC gene* amplicon (217 bp). Lane M 100 bp DNA Ladder E1-E4 represent E. coli isolates, K1-K4 represent Klebsiella pneumoniae and P1-P4 represent P. aeruginosa isolates.

cusCBFA efflux complexes are essential for multiple antimicrobial resistance and to toxic heavy-metal ions and deletion of *cusC* lead to a strong decrease in cooper resistance[52,53]. It is regards as virulence factor due to their responsibility for expelling toxic heavy metals especially Cu(I) and Ag(I) ions[54].

Conclusion:

The results of current study conclude high concentration of most of heavy metals among waste water and emergence multidrug resistance and heavy metals resistance bacteria and Candida spp. and this resistance may attributed to presence of heavy metals and drug efflux pump among those microbes especially CusCFBA pump among Gram-negative isolates.

References:

1. Tilley, E., Ulrich, L., Lüthi, C., Reymond, P. and Zurbrügg, C. (2014). Compendium of sanitation systems and technologies: Swiss Federal Institute of Aquatic Science and Technology (Eawag); Duebendorf, Switzerland; p. 175. ISBN 978-3-906484-57-0.
2. Henze, D. and Buzsáki, G. (2001). Action potential threshold of hippocampal pyramidal cells in vivo is increased by recent spiking activity. Neuroscience., 105(1), pp. 121–30.
3. Subramani T, Mangaiyarkarasi M and Kathirvel C. Impact of Sewage and Industrial Effluent on Soil Plant Health Act on Environment. Int. Journal of Engineering Research and Applications. 2014; 4(6): 270-73.
4. Norup, J. and Aberg, E. Quality assessment of sludge from Glen Valley wastewater treatment plant and its potential as fertilizer. Water and Environmental Engineering Department of Chemical Engineering Lund University, sweeden, 2015 (M.Sc. thesis).
5. Gerardi, M.H. and Zimmerman, M.C. (2005) Wastewater Pathogens. A John Wiley & Sons, Inc Publication. pp. 23-69.
6. Amador, P., Fernandes, R., Prudêncio, M., Barreto, M. and Duarte, I. (2015). Antibiotic resistance in wastewater: Occurrence and fate of Enterobacteriaceae producers of class A and class C β-lactamases. Journal of environmental science and health. Part A, Toxic/hazardous substances & environmental engineering., 50(1), pp. 26–39.
7. Prabhu, S. and Poulose, E.K. (2012). Silver nanoparticles: Mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. International Nano Letters, 2(1):32.
8. Chaturvedi, K.S., Henderson, J.P.(2014).Pathogenic adaptations to host-derived antibacterial copper. Front. Cell. Infect. Microbiol. 4:3.
9. American Puplic Health Association (APHA) . (1985) . Standard Method for the examination of water and wastewater .16th .Ed . (APHA Washington . D.C.) .

10. Goldman, E. and Green, L.H. (2008). Practical Handbook of Microbiology, Second Edition (Google eBook) (Second ed.). USA: CRC Press, Taylor and Francis Group. p. 864. ISBN 978-0-8493-9365-5.
11. Mulamattathil, S.G., Bezuidenhout, C. and Ateba, C.N. (2014). Isolation of Environmental Bacteria from Surface and Drinking Water in Mafikeng, South Africa, and Characterization Using Their Antibiotic Resistance Profiles. Journal of Pathogens, Volume 2014, Article ID 371208, 11 pages
12. Hara-Kudo, Y., Nishina, T., Nakagawa, H., Konuma, H., Hasegawa, J. and Kumagai, S. (2001) 'Improved method for detection of Vibrio parahaemolyticus in seafood', Applied and environmental microbiology., 67(12), pp. 5819–23.
13. Jabra-Rizk, M.A., Brenner, T.M., Romagnoli, M., Merz, W.G., Falkler, W.A., Meiller, T.F. and A. A. M. A. Baqui (2001) 'Evaluation of a reformulated CHROMagar Candida. J ClinMicrobiol.; 39(5): 2015–2016.
14. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. 26th ed. CLSI supplement M100S (ISBN 1-56238-923-8 [Print]; ISBN 1-56238-924-6 [Electronic]). Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500,Wayne, Pennsylvania 19087 USA, 2016.
15. Al-Alaq, F.T., Abdulazeem, L., Al-Dahmoshi, H.O.M., Al-Khafaji, N.S. and Al-Wesawei, Y.A., 2016. PCR-based investigation of oxygenase among crude oil degrading bacteria in Hilla city, Iraq. International Journal of Pharm Tech Research, 9(5), pp.284-291.
16. Al-Dahmoshi, H.O., Almamoori, A.M. and Al-Khafaji, N.S. (2017). Investigation of CusCFBA Pump among Uropathogenic Escherichia coli (UPEC) Isolated from Women with Cystitis, Iraq. Bioscience, Biotechnology Research Asia; 14(1).
17. Park, K. (2005). Hospital Waste Management. Park's Textbook of Preventive and Social Medicine. M/s BanarasidasBhanot Publications. New Delhi. 18th Eds; 595-598.
18. Gupta, S. and Boojh, R. (2007) 'Report: Biomedical waste management practices at Balrampur hospital, Lucknow, India', Waste management &research : the journal of the International Solid Wastes and Public Cleansing Association, ISWA., 24(6), pp. 584–91.
19. Singh, V.S., Gautam, B. and Jag, J.S. (2007). Biomedical Waste Management - An Emerging Concern in Indian Hospitals,1: 1-12.
20. Razdan, P. and Cheema, A.S. (2009). Proceedings of ASCNT. CDAC. Noida. India 2009; 26 – 31.
21. Blumenthal, U.J., Mara, D.D., Peasey, A., Ruiz-Palacios, G. and Stott, R. (2000). Guidelines for the microbiological quality of treated wastewater used in agriculture: Recommendations for revising WHO guidelines. Bulletin of the World Health Organization, 78 (9):1104-1116.
22. Yang, C.M., Lin, M.F., Liao, P.C., Yeh, H.W., Chang, B.V., Tang, T.K., Cheng, C., Sung, C.H. and 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), pp. 560–565.
23. Filipkowska, Z., Gotkowska-Plachta, A. and Korzeniewska, E. (2008). Moulds, yeasts and yeast-like fungi in the atmospheric air at constructed wetland systems (with aerated and stabilization ponds) and in the surrounding area. Water-Environment-Rural Areas, 8(1), pp.69-82.
24. Biedunkiewicz, A. and Ozimek, T. (2009). Qualitative and quantitative changes of potentially pathogenic fungi in a hydrophyte wastewater treatment plant. Polish Journal of Environmental Studies, 18(2), pp.161-166.
25. Korzeniewska, E., Filipkowska, Z., Gotkowska-Płachta, A., Janczukowicz, W., Dixon, B. and Czułowska, M. (2009). Determination of emitted airborne microorganisms from a BIO-PAK wastewater treatment plant. Water research, 43(11), pp.2841-2851.
26. Howard D. H. Pathogenic Fungi in Humans and Animals. 2nd ed. Mycology series Vol. 16. ed. J. W. Bennett. Marcel Dekker, Inc. New York, Basel. pp. 790, 2003.
27. Naglik, J., Challacombe, S. and Hube, B. (2003) 'Candida albicans secreted aspartyl proteinases in virulence and pathogenesis', Microbiology and molecular biology reviews : MMBR., 67(3), pp. 400–28.
28. Blaak, H., Lynch, G., Italiaander, R., Hamidjaja, R.A., Schets, F.M. and de RodaHusman, A.M. (2015) 'Multidrug-Resistant and extended spectrum Beta-Lactamase-Producing Escherichia coli in Dutch surface water and wastewater', PLOS ONE, 10(6), p. e0127752
29. Cornejova, T., Venglovsky, J., Gregova, G., Kmetova, M. and Kmet, V. (2015). Extended spectrum beta-lactamases in Escherichia coli from municipal wastewater. Ann Agric Environ Med.; 22(3): 447–450.
30. Rupinder, B., Geeta, W. and Shikha, J. (2013). Prevalence of extended spectrum β-lactamases in multidrug resistant strains of gram negative Bacilli. J. Acad. Indus. Res. ;1(9):558–560.

31. Abhijit, A., Sunita, N. and Maria, K. (2013). Study of urinary isolates with reference to extended spectrum beta lactamases detection and antibiogram. J. Evol. Med. Dent. Sci. ;2(9):1049–1055.
32. Majda, Q., Najma, A. and Summyia, B. (2013). Evaluation of extended spectrum beta-lactamase mediated resistance in Escherichia coli and Klebsiella in urinary tract infection at a tertiary care hospital. Biomedica. ;29:78–81.
33. Meeta, S., Sati, P. and Preeti, S. (2013). Prevalence and antibiogram of extended spectrum β-lactamase (ESBL) producing Gram negative bacilli and further molecular characterization of ESBL producing Escherichia coli and Klebsiella spp. J. Clin. Diag. Res. ;7(10):2168–2172.
34. Kritu, P., Prakash, G., Shiba, K.R., Reena, K.M., Ram, N.S. and Ganesh, R. (2013). Antibiogram typing of gram negative isolates in different clinical samples of a tertiary hospital. Asian J. Pharm. Clin. Res. ;6(1):153–156.
35. White, P.A., McIver, C.J. and Rawlinson, W.D. (2001). Integrons and Gene Cassettes in theEnterobacteriaceae. Antimicrobial agents and chemotherapy, 45(9), pp.2658-2661.
36. Singh, R., Schroeder, C.M., Meng, J., White, D.G., McDermott, P.F., Wagner, D.D., Yang, H., Simjee, S., DebRoy, C., Walker, R.D. and Zhao, S., (2005). Identification of antimicrobial resistance and class 1 integrons in Shiga toxin-producing Escherichia coli recovered from humans and food animals. Journal of Antimicrobial Chemotherapy, 56(1), pp.216-219.
37. Rizzo, L., Fiorentino, A., and Anselmo, A. (2012). Effect of solar radiation on multidrug resistant E. coli strains and antibiotic mixture photodegradation in wastewater polluted stream. Sci. Total Environ.427–428:263–268.
38. Rahube, T.O., Viana, L.S., Koraimann, G., and Yost, C.K. (2014). Characterization and comparative analysis of antibiotic resistance plasmids isolated from a wastewater treatment plant. Front. Microbiol.5:558.
39. Talebi, M., Rahimi, F., Katouli, M., Möllby, R. and Pourshafie, M.R., (2008). Epidemiological link between wastewater and human vancomycin-resistant Enterococcus faecium isolates. Current microbiology, 56(5), pp.468-473.
40. Araújo, C., Torres, C., Silva, N., Carneiro, C., Gonçalves, A., Radhouani, H., Correia, S., da Costa, P.M., Paccheco, R., Zarazaga, M., Ruiz-Larrea, F., Poeta, P. and Igrejas, G. (2010) 'Vancomycin-resistant enterococci from Portuguese wastewater treatment plants', Journal of Basic Microbiology, 50(6), pp. 605–609
41. Goldstein, R., Micallef, S., Gibbs, S., George, A., Claye, E., Sapkota, A., Joseph, S. and Sapkota, A. (2013). Detection of vancomycin-resistant enterococci (VRE) at four U.S. Wastewater treatment plants that provide effluent for reuse', The Science of the total environment., 466, pp. 404–11.
42. Jiang, L., Hu, X., Xu, T., Zhang, H., Sheng, D. and Yin, D. (2013) 'Prevalence of antibiotic resistance genes and their relationship with antibiotics in the Huangpu river and the drinking water sources, Shanghai, china', Science of The Total Environment, s 458–460, pp. 267–272.
43. Moges, F., Endris, M., Belyhun, Y. and Worku, W. (2014) 'Isolation and characterization of multiple drug resistance bacterial pathogens from waste water in hospital and non-hospital environments, northwest Ethiopia', 7.
44. Guo, X., Li, J., Yang, F., Yang, J. and Yin, D. (2014) 'Prevalence of sulfonamide and tetracycline resistance genes in drinking water treatment plants in the Yangtze river delta, china', Science of The Total Environment, 493, pp. 626–631.
45. Kafilzadeh, F., Moghtaderi, Y. and Jahromi, A.R. (2013) Isolation and Identification of Cadmium-Resistant Bacteria in Soltan Abad River Sediments and Determination of Tolerance of Bacteria through MIC and MBC. European Journal of Experimental Biology, 3, 268-273.
46. Devika, L., Rajaram, R. and Mathivanan, K. (2013) Multiple Heavy Metal and Antibiotic Tolerance Bacteria Isolated from Equatorial Indian Ocean. International Journal of Microbiological Research, 4, 212-218.
47. Novo, A., André, S., Viana, P., Nunes, O.C. and Manaia, C.M. (2013) Antibiotic Resistance, Antimicrobial Residues and Bacterial Community Composition in Urban Wastewater. Water Research, 47, 1875-1887.
48. Shamim, S. and Rehman, A. (2012) Cadmium Resistance and Accumulation Potential of Klebsiella pneumoniae Strain CBL-1 Isolated from Industrial Wastewater. Pakistan Journal Zoology, 44, 203-208.
49. Jafari, A., Aslani, M., and Bouzari, S. (2012). Escherichia coli: a brief review of diarrheagenicpathotypes and their role in diarrheal diseases in Iran. Iranian Journal of Microbiology, 4(3), 102–117.

50. Lyons, C.N. and White, T.C. (2000). Transcriptional analyses of Antifungal drug resistance in Candida albicans. Antimicrob Agents Chemother; 44(9):2296–2303.
51. Karababa, M., Coste, A.T., Rognon, B., Bille, J. and Sanglard, D. (2004). Comparison of gene expression profiles of Candida albicans Azole-Resistant clinical isolates and laboratory strains exposed to drugs inducing Multidrug transporters. Antimicrobial Agents and Chemotherapy, 48(8), pp. 3064–3079.
52. Franke, S., Grass, G., Rensing, C. and Nies, D.H. (2003). Molecular analysis of the copper-transporting efflux system CusCFBA of Escherichia coli. Journal of bacteriology; 185(13):3804-3812.
53. Saier, M.H., Tran, C.V. and Barabote, R.D. (2006). TCDB: the Transporter Classification Database for membrane transport protein analyses and information. Nucleic acids research ; 34(suppl 1):D181-6.
54. Kulathila, R., Kulathila, R., Indic, M. and van den Berg, B. (2011). Crystal Structure of Escherichia coli CusC, the Outer Membrane Component of a Heavy Metal Efflux Pump. PLoS ONE 6(1): e15610.

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