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Prevalence of Antibiotic Resistance Genes (ARGs) in a small wastewater treatment plant in Egypt

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Abstract **-** This study aimed to evaluate the occurrence and abundance of antibiotic resistance genes (ARGs) in one of the rural wastewater treatment plants (WWTP) in Egypt, namely Hawamdya. Conventional PCR and qPCR were used to detect and quantify the abundance of β-lactam (ampC and OXA-1), sulfonamide (sul1 and sul2), tetracycline (tetO and tetW), and macrolide (ermF and ermB) as well as class 1 integron (intl1). DNA was extracted from influent, activated sludge, and effluent samples. ARGs were detected and quantified in all the tested samples using specific primers for each gene, suggesting the prevalence of ARG in the wastewater entering and persisting the WWTP. The intl1 gene, a marker for integrons, was highly abundant in the influent and activated sludge, confirming that integrons play a significant role in the spread of antibiotic resistance in the Hawamdya WWTP. The highest occurrence of sul1 and ermF genes among all samples was detected in influent, suggesting the widespread use of sulfonamide and macrolide antibiotics in the located area. The activated sludge showed comparable copy numbers for most ARGs, suggesting that it serves as a reservoir of antibiotic-resistance genes. The effluent showed a high copy number for sul1 and sul2 genes but a lower level of intl1, indicating that the treatment process partially removes ARGs but may be less effective against integrons. The present study revealed a high abundance of ARG, especially sulfonamide resistance genes, in tested rural WWTP as a point source of ARGs in the environment and emphasized the need to control antibiotic use and develop more effective wastewater treatment strategies to minimize the spread of antibiotic resistance.

Keywords**:** Wastewater Treatment; Antibiotics; Antibiotic-resistance genes; Rural Sanitation, Benchmarking.

1. Introduction

Antimicrobial agents such as antibiotics are one of the most successful strategies used in modern medicine to inhibit human and animal bacterial pathogens. The worldwide increase in antibiotic consumption rate leads to many negative effects, such as increased bacterial resistance to current antibiotics and accumulating residual antibiotics in wastewater from partially absorbed antibiotics [1]. Therefore, different antibiotic-resistance genes (ARGs) which evolved by many pathogenic bacteria to survive, are responsible for the emergence of antibiotic-resistant bacteria (ARBs). In addition, the accumulating residual antibiotics induces bacterial gene mutations, hence the development of ARGs, and hastens the spread of ARGs through the horizontal bacterial gene transfer mechanism, as shown in Figure 1 [2]. The mobile genetic elements (MGE), which transfer the ARGs, may carry one or multiple ARGs [3]. These genes were detected in environments such as soil, feces, surface water, wastewater effluents, and food products.

Worldwide, one of the main anthropogenic release sources of residual antibiotics into the environment is raw wastewater as well as the effluent of wastewater treatment plants (WWTPs). Inside the WWTPs, the processing and treatment of WW initiate a favourable environment for the development of ARGs and the occurrence of HGT, as there is a continuous mixing with antibiotics at sub-inhibitory concentrations [4]. Although the concentrations of detected antibiotics are low, the possible enrichment of resistant bacterial mutants carrying ARGs is high. Recently, several studies have investigated the prevalence of antibiotic resistance genes (ARGs) in wastewater treatment plants (WWTPs), particularly large facilities serving urban populations. However, research is remarkably scarce regarding the prevalence of ARGs in smaller WWTPs catering specifically to rural areas. This limited understanding poses a significant knowledge gap, as sustainability challenges

in rural sanitation systems are often more pronounced, especially in low-income countries like Egypt [5]. Conventional wastewater treatment used in these rural areas, encompassing dilution, physicochemical, and biological methods, faces several limitations [6]. Furthermore, treatment facilities in many rural areas across diverse countries typically operate with less stringent effluent quality restrictions [7], raising crucial questions about the levels of antibiotics and ARGs present in the discharged effluent from these smaller WWTPs. They struggle to eliminate the wide range of pollutants present, contribute to high energy consumption, fail to achieve complete pollutant removal, and generate toxic sludge as a byproduct.

The occurrence of ARGs as a new contaminant in treated wastewater without an effective treatment method increases the possible transfer of ARGs to the food chain, such as aquatic products, poultry meats, fermented foods, and livestock. The significance of ARG in environmental systems and food chain increases the risk of harmful effects on human health [1]. The correlation between the shared ARGs from environmental bacteria and human pathogens was reported. Indeed, no direct evidence confirms the transmission of ARGs from food to natural bacterial flora in the gut of humans and animals. It is worth noting that the determination of the start of the dissemination of ARGs and the accumulation rate of ARGs in the wastewater network became crucial. There are two main approaches to assessing the accumulation of ARGs, either using culture-based or molecular-based approaches. Recently, molecular-based approaches have been spread to quantify the ARGs. Especially, the quantitative polymerase chain reaction (qPCR) which has been used extensively in the assessment of ARGs [8].

This study aims to monitor the prevalence of ARGs in Egyptian WWTPs using a conventional and real-time PCR method. Further, the gene copy number quantification of nine different ARGs (sul1, sul2, ampC, intl1, tetW, tetO, oxa-1, ermF, and ermB) in the Hawamdya WWTPs among the influent, activated sludge, and effluent.

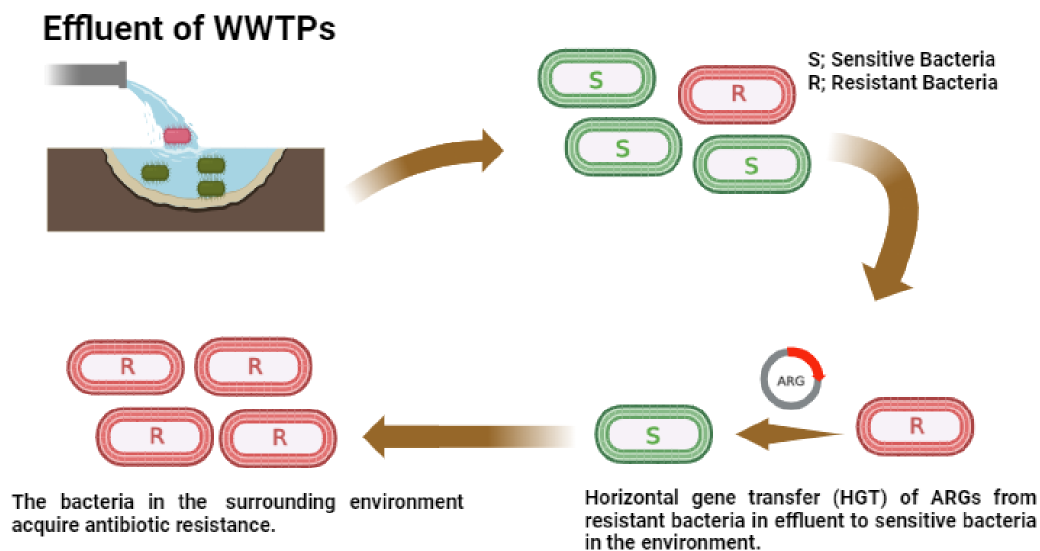

Figure 1: Diagram showing the horizontal gene transfer that spread the antibiotic resistance to the environment.

2. Experimental Methods

2.1. Wastewater treatment plant description

Hawamdya WWTP is considered a small WWTP, located in the rural area of Giza governorate, and has a capacity of 20000 m^3/d. The first phase of Hawamdya WWTP was commissioned in 2009, and there is an ongoing extension to reach 40,000 m^3/d. The treatment process of the existing WWTP is trickling filters. The process units mainly consist of a coarse screen, grit removal, primary sedimentation tanks, trickling filters, final sedimentation tanks, and chlorine contact tanks. The sludge is conveyed to thickeners, followed by drying beds. The WWTP serves rural areas and villages in the Giza governorate. A description of the influent and effluent characteristics is included in Table 1.

2.2. Samples collection

For sampling, 2 hours of composite sampling were collected during the morning period, and a mixture of 5 samples was taken every 30 minutes over the 2 hours. All the sampled material is gathered in one container throughout the sampling

period. This data, collected over time, will represent a wastewater treatment plant's typical performance during that time. Collected samples were stored at 4° C before further analyses were performed.

Table 1: Capacities and Average Wastewater Analysis for Hawamdya WWTP

WWTP	Capacity (m3/d)	Average Influent			Average Effluent		
		COD, mg/l	BOD, mg/l	TSS, mg/l	COD, mg/l	BOD, mg/l	TSS, mg/l
Hawamdya WWTP	20,000	600	500	500	80	50	50

2.3 DNA extraction method

The genomic DNA was extracted from the collected samples of Hawamdya WWTP using a Genomic DNA Kit (GeneJET ™, Thermo Scientific, USA) following the kit's instructions. Additional steps were performed in the DNA extraction method to improve the quality and yield of the extracted DNA. In the lysis step, 0.5 g of sterile sand beads (diameter = 0.2 mm) were mixed with the precipitate of each sample, and the suspensions were mixed by vortexing for one minute. The obtained suspension was mixed with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) to remove any contaminating proteins, fats, and cells debris; then, the mixture was centrifugated at 14000 rpm for 5 min. The DNA's aqueous upper layer was transferred to a new Eppendorf tube. Finally, the DNA was harvested and then washed as described in the kit's instructions. DNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies/Thermo Scientific) via absorbance measurements at 260 and 280 nm wavelengths. The purity of the extracted DNA was determined by checking the ratio of A_{260}/A_{280} as an indicator of the purity of the DNA. The integrity of the obtained DNA samples was evaluated by agarose gel electrophoresis.

2.4. Conventional PCR

Conventional PCR assays were performed to detect the occurrence of the nine selected ARGs (*sul*1, *sul*2, *amp*C, *intl*1, *tet*W, *tet*O, *oxa*-1, *erm*F, and *erm*B). The PCR reactions were carried out using the 2x master mix (amaR OnePCR™, GeneDireX, Inc.) in a total 20 µl volume reaction. The PCR reactions contain 1 µl from each primer 10 µM (forward and reverse), and 1 µl of template 50 ng. The optimal conditions (concentration and annealing temperature) were optimized guided by the published data, where the temperature varies according to each primer for ARGs genes as shown in Table 2. The temperature program consisted of initial denaturing at 95°C, followed by 30 cycles of 30 s at 95°C; 30 s at the annealing temperature (showed in Table 2); 30 s at 72°C, and a final extension step for 10 min at 72°C. The PCR thermal cycler used was Wee32® (HiMedia Laboratories Pvt Ltd., India). The amplicons were finally visualized after agarose gel electrophoresis in 1.5% agarose gel and photographed using the Gel Doc™ Imager system (Bio-Rad).

Table 2: Table showing the primers information.

Gene Target	Antibiotic	Primer Sequence (5'→3')	Size (bp)	Annealing Temp. (°C)	References
*amp*C	β-lactam	**F**: CCT CTT GCT CCA CAT TTG CT **R**: ACA ACG TTT GCT GTG TGA CG	189	58	[9]
OXA-1	β-lactam	**F**: TAT CTA CAG CAG CGC CAG TG **R**: CGC ATC AAA TGC CAT AAG TG	199	60	[9]
*sul*1	sulfonamide	**F**: CGC ACC GGA AAC ATC GCT GCA C **R**: TGA AGT TCC GCC GCA AGG CTC G	163	58	[10]
*sul*2	sulfonamide	**F**: TCC GGT GGA GGC CGG TAT CTG G **R**: CGG GAA TGC CAT CTG CCT TGA G	191	58	[10]
*intl*1	integron	**F**: GCC TTG ATG TTA CCC GAG AG **R**: GAT CGG TCG AAT GCG TGT	409	60	[11]
*tet*O	tetracycline	**F**: ACG GAR AGT TTA TTG TAT ACC **R**: TGG CGT ATC TAT AAT GTT GAC	171	50	[12]
*tet*W	tetracycline	**F**: GAG AGC CTG CTA TAT GCC AGC **R**: GGG CGT ATC CAC AAT GTT AAC	168	64	[12]

*erm*F	macrolide	**F**: CGA CAC AGC TTT GGT TGA AC **R**: GGA CCT ACC TCA TAG ACA AG	309	56	[13]
*erm*B	macrolide	**F**: GAT ACC GTT TAC GAA ATT GG **R**: GAA TCG AGA CTT GAG TGT GC	364	58	[13]

2.5. Quantitative real-time (qPCR) for the ARGs

In order to plot the calibration curve, the amplified PCR fragments were extracted using DNA Gel Extraction Kit (Norgen Biotek Corp., Thorold, Canada) according to the kit's instructions. The purified PCR fragments concentrations were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies/Thermo Scientific) via absorbance measurements at 260/280 nm. Then, the purified PCR fragments of the optimized nine ARGs; *sul*1, *sul*2, *amp*C, *intl*1, *tet*W, *tet*O, *oxa*-1, *erm*F, and *erm*B, from 1 to 10^{-7} dilution fold with 10-fold dilution. Calibrators were diluted in nuclease-free water (ThermoFisher Scientific, USA) and used as a real-time PCR template. For each gene, at least four serial concentrations of the purified PCR amplicons were used for the subsequent qPCR. The reactions were performed in triplicate, to produce a calibration curve.

qPCR protocols were carried out using the Maxima SYBR Green qPCR Master Mix 2X (ThermoFisher Scientific, USA). Reactions were performed in 0.2 ml qPCR 8-strip tubes (Gunster Biotech, Taiwan) using a Bio-Rad CFX96 (Bio-Rad Laboratories, Inc., Germany). Each qPCR reaction contained 10 µl of 2X Maxima SYBR Green Mix, 0.2 µM final concentration of primer (for each forward and reverse), and 1 µl of the DNA template (all samples diluted to 4 ng/µl). The three samples from Hawamdya WWTP (I; Influent, A; Activated Sludge, and E; Effluent) were used as a template for triplicate amplification. The qPCR program was as follow; initial denaturing at 95°C for 10 min, followed by 40 cycles of 30 s at 95°C; 30 s at the annealing temperature (the temperature varies according to each primer for ARGs genes, see Table 2); 30 s at 72°C, and a final extension step for 10 min at 72°C using. The melting curves were generated as follows; 95°C for 10 s, 65°C for 60 s, 97°C for 0.5 s. Following qPCR amplification of the standards and samples, the raw Cq data was imported, and the calibration curve plotted.

The copy number per 4 ng of DNA of the nine ARGs were quantified using the method of Tichopad and his colleagues [14]. The equation for calculating the initial number of copies/sample is $(X_o) = E_{AMP}^{(b-Cq)}$ wherein E_{AMP} (exponential amplification efficiency which is $10^{(-1/slope)}$) and b = the y-intercept of the standard curve [15].

3. Results

The analysis of genomic DNA demonstrated that the modified method produces a relatively high yield ranged from 65 to 100 ng/ul with an accepted purity (Table 3). The quality of the extracted DNA showed that all samples have good integrity (Figure 1). As expected, the DNA extracted from the influent and the activated sludge had higher concentrations than the effluent samples.

Table 3: The concentrations and purity of the extracted DNA Hawamdya (I; Influent, A; Activated Sludge, and E; Effluent).

Sample	I	A	E
Concentrations (ng/ul)	100	80	65
Purity (A260/A280)	1.65	1.79	1.61

The occurrence of antibiotic families; β-lactam (ampC and OXA-1 genes), sulfonamide (sul1 and sul2 genes), tetracycline (tetO and tetW genes), macrolide (ermF and ermB genes) and integron (intl1 gene) were tested using the conventional PCR. Figure 2 demonstrated that all PCR reactions were successfully detected in the WWTP. The amplicon size for intl1 gene was 409 bp, tetO was 171 bp, tetW was 168, ermB was 364 bp, ermF was 309, sul1 was 163, sul2 was 191, ampC was 189 and OXA-1 was 199.

In order to generate calibrators for the qPCR, the amplicons of the above-mentioned genes were successfully extracted and purified. The generated calibrators with different dilutions were used for the plotting of the standard curves for each gene using the qPCR. The generated standard curves were used to determine the efficiency of the qPCR reaction for each gene. The efficiency of the standard curves was acceptable and ranged from 81.41 - 124.75 percentage. From the standard curves the intercept was calculated for each gene to determine the copy number for each sample.

On the other hand, melt curve analysis was performed to examine the specificity of the primer for each tested sample. The melt curve profiles were almost the same for all the samples. Taken together, the melt curve seemed to assure the single product of the qPCR reactions. Therefore, the comparison of fluorescent amplification curves resulted in valid raw Cq data.

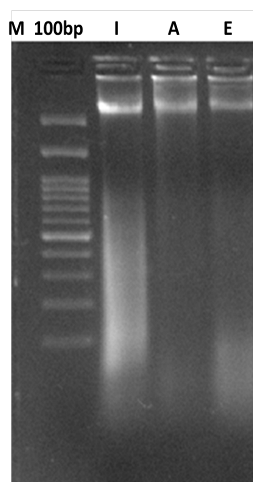

Figure 1: DNA gel electrophoresis of DNA samples after the DNA extraction. M; 100 bp DNA ladder; I; Influent, A; Activated Sludge, and E; Effluent.

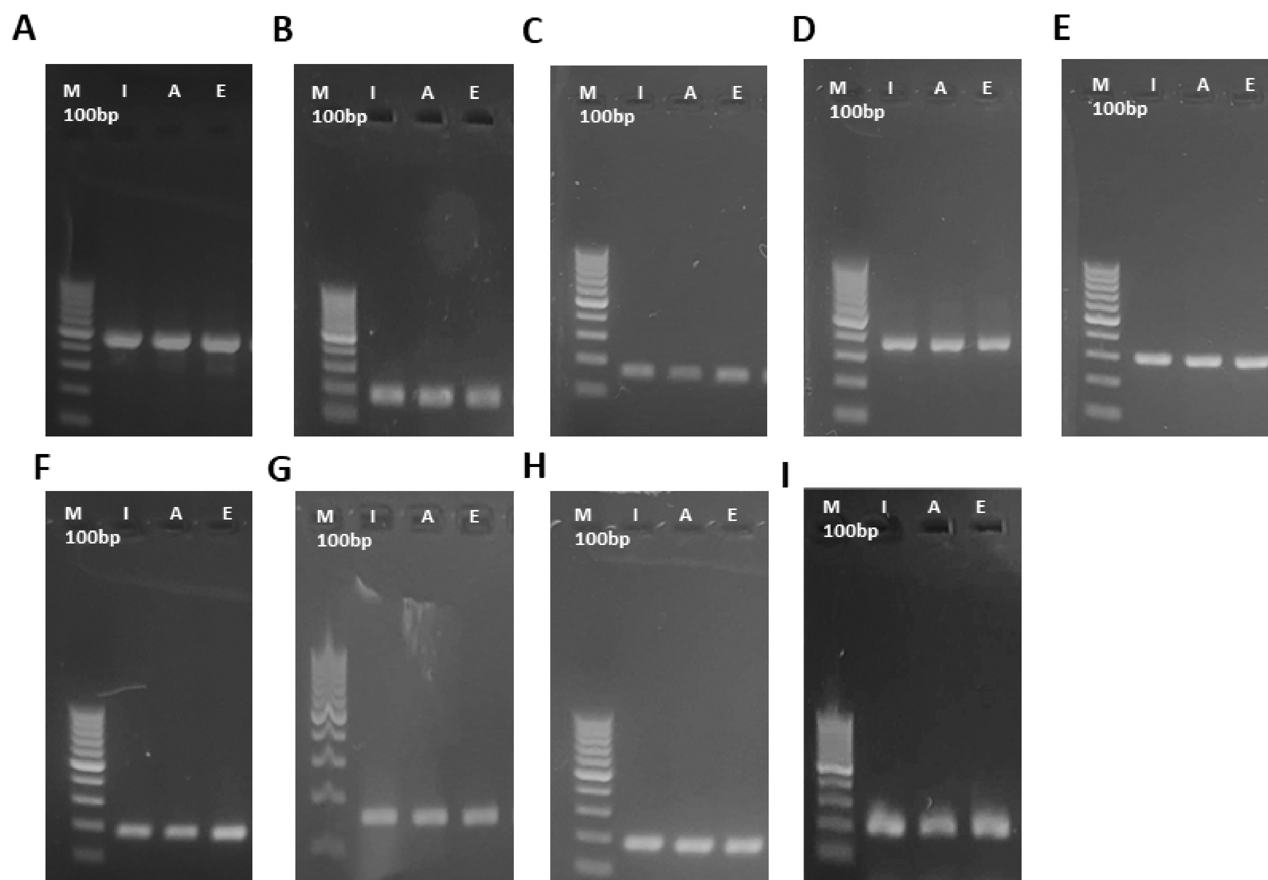

Figure 2: Agarose gel electrophoresis shows the occurrence patterns of different ARGs as analyzed by PCR. Lane A; intl1, B; tetO, C; tetW, D; ermB, E; ermF, F; ampC, G; sul1, H; sul2, and I; oxa-1. The PCR performed on samples taken from Hawamdya WWTP (I; Influent, A; Activated Sludge, and E; Effluent).

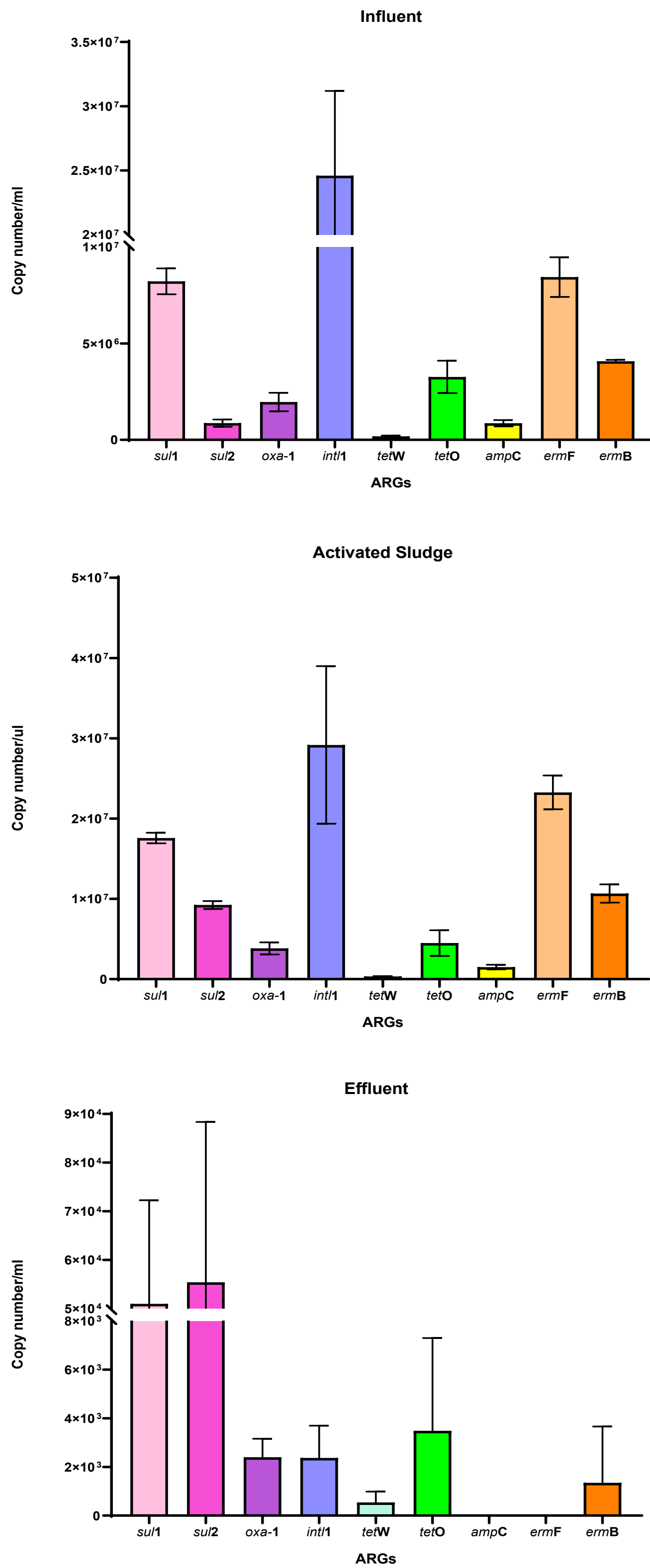

Figure 3: Gene copy number per 4 ng of DNA for the nine ARGs in Hawamdya WWTP in influent, activated Sludge, and effluent

qPCR- detectable antibiotic families, including β-lactam (*amp*C and *OXA*-1), sulfonamide (*sul*1 and *sul*2), , tetracycline (*tet*O and *tet*W), macrolide (*erm*F and *erm*B) genes as well as integron (*intl*1) associated with mobile elements of DNA were determined in each sample (Influent, Activated Sludge, and Effluent). In the influent, the intl1 gene was the most abundant gene, and a high copy number of sul1 and ermF genes were detected (Figure 3). Remarkably, the dominance of the integron resistance gene (intl1) in the selected WWTP was in line with previous literature [16], [17], [18]. In the activated sludge of the Hawamdya WWTP, almost all the selected genes showed a comparable copy number. The effluent of the Hawamdya WWTP recorded a high copy number for the sulfonamide (*sul*1 and *sul*2) genes, but the *int*l gene revealed a lower level.

4. Conclusion

The present study recorded a high prevalence of ARGs families in the rural Hawamdya WWTP. The occurrence of different ARG families, including β-lactam, sulfonamide, tetracycline, and macrolide, were abundant after the water treatment in the effluent samples. Furthermore, the dominance of the integron resistance gene (intl1), related to gene transfer from bacterium to bacterium, explains the spread of ARG within the WWTP. This study suggests that conventional wastewater treatment using trickling filters is ineffective in the removal of ARGs.

Acknowledgments

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