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RESEARCH ARTICLE

Multi-Drug Resistant Bacteria from Sewage Treatment Plants in Johor: Isolation and Characterization

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Abstract In this study, we revealed multi-drug resistant (MDR) bacteria isolated from three sewage treatment plants (STPs) against ciprofloxacin, chloramphenicol, gentamicin, tetracycline, and sulfamethoxazole. The antibiotic susceptibility test (AST) result shows that these isolates are distinctly highly resistant to sulfamethoxazole for influent (100%) and effluent (80-100%) samples for the first sampling (S1), while the lowest resistance (0%), resistant to chloramphenicol in some locations, in the second sampling (S2). Among the culturable isolates, multi-resistant bacteria were screened through AST, and these species were identified through 16S rRNA gene sequencing. From the cumulative multi-resistant isolates, 45.45% are known opportunistic bacteria species from the *Enterobacteriaceae* family (*Citrobacter sp*., *Serratia sp*., *Enterococcus sp*., and *Escherichia sp*.), while 27.27% are *Aeromonadaceae* and *Pseudomonadaceae*, respectively. This study reveals the prevalence of culturable multi-resistant opportunistic bacteria in influent and effluents of the three selected STPs for both sampling times.

Keywords: Antibiotic susceptibility; antibiotic resistant bacteria; STP – sewage treatment plants; pathogen; MAR Index.

Introduction

Antimicrobial resistance (AMR) is recognized to threaten human health and the environment globally, and the emergence of multi-resistant pathogens has raised a severe clinical concern [1]. Antibiotics found in wastewater can contribute to the emergence of drug-resistant bacteria, as their sub-lethal concentrations are insufficient to kill bacteria but can influence microbial genetic and phenotypic diversity [26]. A significant public health concern arises from the potential transfer of resistance genes from environmental bacteria to human pathogens. This gene exchange is particularly likely to occur in STPs and nearby aquatic environments, which provide favourable conditions for such events [26]. Among all environmental compartments, aquatic ecosystems have been identified as the primary reservoirs of antibiotic-resistant bacteria (ARB) [26]. The spread of AMR to the surface water contributes to further broadening the sphere of AMR and the spread of resistance traits. Since the 20th century, the consumption of antibiotics has gained substantial acknowledgment. At the same time, the study on the development of antibiotic resistance is equally significant. In 2019, around 1.27 million deaths were associated with AMR, and millions were hospitalized and suffered prolonged sickness [2]. This silent pandemic may result in up to ten million casualties per year by 2050 if no appropriate action is taken immediately against the rapid growth of AMR [2].

Huge concern has been raised recently due to the emergence of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) in the environment caused by the disproportionate and improper use of several antibiotics [3]. Horizontal gene transfer (HGT) of new resistance attributes in a diverse bacterial environment may result in carriers with an immense pool of ARGs among the same species and more diversified bacteria [7]. Thus, resulting in ARGs inside biological treatment facilities are not adequately eradicated and can even be increased [8].

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Wastewater treatment plants (WWTPs) provide ideal conditions for the proliferation of ARB and horizontal transfer of ARGs among various microorganisms [6]. Bacteria abundant in WWTPs are responsible for breaking down organic matter, providing nutrient sources, and aiding in energy flow and biogeochemical cycling [4]. The situation is particularly alarming when multi-resistant bacteria, pathogens, and opportunistic microorganisms in this environment evolve to a condition where antibiotics are no longer effective in treating infections. The cases of untreatable multidrug-resistant bacteria that are resistant to regular antibiotics due to the development of antimicrobial resistance may lead to a global healthcare crisis when bacterial resistance becomes more common [9].

Conventional wastewater treatment processes are reported to be able to remove some ARB, but a sizable amount of ARB can still be found in the effluent. As a result, these bacteria carrying resistance traits can still be released, reaching downstream habitats and thus are broadly circulated in the environment [10]. WWTPs play substantial roles in balancing the overall health of the ecosystem [12]. Thus, when not properly managed, they may cause changes in the distribution and quantity of bacteria carrying resistance genes in WWTPs, and the release of pathogens to receiving water may threaten biological balance and safety [13].

The objective and significance of this study align with the Sustainable Development Goals (SDG) by the United Nations (UN), particularly focusing on "good health and well-being" and "clean water and sanitation". Polymerase Chain Reaction (PCR)-based methods, independent of cultivation, defined the presence of ARGs in bacteria for resistant isolates, and this method has been widely used by researchers [2]. However, these methods fail to relate these microorganisms to clinical pathogen susceptibility levels (susceptible, intermediate, resistant) [2]. Bacteria's direct isolation from the environment offers a realistic risk assessment compared to molecular analysis, such as metagenomics alone [13]. Due to bacterial DNA alterations, researchers have recently had to undertake ongoing surveillance operations for resistance patterns [14]. Specific pathogen resistance and sensitivity identified through laboratory culturing procedures are essential for healthcare providers to promptly institute appropriate treatment regimens for patients [15]. Therefore, the primary goal of this investigation is to identify the antibiotic susceptibility of the prevalent multi-resistant bacteria in the influent and effluent of three STPs located in Johor.

Materials and Methods

Materials and Sampling Locations

Sewage samples were collected from three different STPs under Indah Water Konsortium Sdn Bhd, located in Johor, Malaysia. The samplings were conducted twice for the three locations for S1 (sampling in October 2018) and S2 (sampling in April 2019), respectively. The STPs involved are Taman Harmoni (TH), Taman Selesa (TS), and Bandar Putra (BP) for the first sampling (S1) and Taman Harmoni2 (TH2), Taman Selesa2 (TS2), and Bandar Putra2 (BP2) for the second sampling (S2). The three selected STPs in this study were decentralized Extended Aeration STPs. These STPs consist of treatment stages from influent to screening, aeration, and secondary clarifier, then to the final effluent. Two different sampling points, which are the influent sample and the effluent sample, were taken from each location. The sewage samples were collected in amber bottles and transferred on ice to preserve the cultures.

Isolation of Bacterial Isolates

The sewage samples were diluted with sterile distilled water to make bacterial suspensions. From the suspension, 100µL of the aliquot was spread on nutrient agar (NA) and incubated at 37°C overnight to obtain mixed colonies of bacterial cultures on NA plates. Each colony present was streaked individually to obtain pure isolates. Each bacterium present in each plate was then streaked a few times in NA until pure cultures were obtained from each NA plate. All isolates are labelled based on locations and sampling points, which are influent (I) and effluent (E) accordingly.

Antibiotic Susceptibility Test (AST)

The AST was performed using the Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines to determine bacteria sensitivity and resistance [16]. The bacterial colonies were grown overnight with an incubation temperature of 37°C. The overnight culture inoculum was used to prepare a direct bacterial suspension and was standardized to a 0.5 McFarland suspension turbidity [15]. Respective bacterial isolates with 0.5 McFarland suspension turbidity were then streaked onto Mueller-Hinton agar (MHA) (Oxoid, UK), followed by placing the antibiotic disks on MHA. A total of 5 antibiotics were tested for antibiotic susceptibility of each isolate by application of antibiotic disks on inoculated MHA plates for each isolate. Disk diffusion methods of antibiotics were performed for antibiotics ciprofloxacin (5µg), chloramphenicol (30µg), gentamicin (10µg), tetracycline (30µg), and sulfamethoxazole (300µg), respectively, for each bacterial isolate. All antibiotics are from the brand Oxoid, UK. The plates were again incubated for 18-20 hours at 37°C, and then the growth inhibition zones for respective antibiotics were measured by diameter, and the results were compared to the breakpoint value according to CLSI for interpretation of result to determine the resistance.

Identification of Multi-Resistant Isolates

The bacteria MAR index was determined using the formula MAR = *a/b*, where *a* represents the antibiotics these isolates are resistant to, while *b* represents the total number of antibiotics tested for AST [17]. A MAR Index value of 0.2 or higher indicates exposure to high-risk environments with frequent antibiotic usage [5]. The DNA of bacteria with MAR index ≥ 0.2 was extracted using a simple boiling method [18] and amplified using the polymerase chain reaction (PCR) method using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The purity of the extracted DNA was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA). Then, gel electrophoresis for all extracted DNA was performed to identify the success of 16S rRNA gene amplification [19] before sending these samples for 16S rRNA gene sequencing bacteria identification. The extracted bacterial DNA was sent to Apical Scientific Sdn. Bhd. for 16S rRNA gene sequencing. Nucleotide sequences from 16S rRNA gene sequencing were analyzed using DNA Chromatogram Explorer. Then the data were aligned using ClustalW and compared to GenBank, 16S rRNA genes [20].

Results and Discussion

Details of Sampling Location(s) and Number of Bacteria Isolated from Selected STPs

Table 1 shows the total number of 50 isolates isolated from the traditional culturing method from influent and effluent samples collected at three selected STP locations (TH, TS, and BP) during two sampling durations, S1 (October 2018) and S2 (April 2019). The total number of culturable bacterial isolates reflects consistent bacterial presence throughout the wastewater treatment process. These findings align with similar investigations that demonstrate wastewater contains diverse microbial communities that often survive conventional treatment processes to varying degrees.

At TH (JKI060), eight isolates were obtained during both sampling periods, although the number of bacterial isolates isolated between influent and effluent varied. Influent isolates increased from three in S1 to five in S2, while effluent isolates decreased from five to three. The variation in the number of bacteria isolated shows temporal changes in bacterial occurrence, potentially influenced by influent composition or treatment performance. Similar temporal fluctuations in bacterial recovery have been documented in WWTPs and are often attributed to treatment processes and operational conditions [48]. The TS site (JKI052) showed the greatest temporal variation, with total isolates increasing from five in S1 to eleven in S2. The increased number of isolates in S2, including six from effluent samples, indicates enhanced bacterial persistence during the later sampling period. At BP (JKI065), the total number of isolates remained constant across both sampling periods (nine isolates each). Effluent samples consistently yielded slightly more isolates than influent samples, suggesting the persistence of bacterial populations through the treatment process.

This finding is consistent with reports indicating that conventional biological treatment processes reduce bacterial abundance but do not completely eliminate microorganisms [45]. Overall, the consistent presence of bacteria from effluent samples across all sites demonstrates that conventional wastewater treatment processes were insufficient to completely remove culturable bacteria.

Table 1. Details of sampling location(s) and number of bacteria isolated from TS, TH and BP from influent samples and effluent samples in S1 and S2 respectively.

Sample Details			Number of Bacteria Isolated			
Sampling Location (Coordinate)	**Asset Code**	**Population Equivalent**	**Sampling Time (S1/S2)**	**Number of Bacteria Isolated from Influent**	**Number of Bacteria Isolated from Effluent**	**Total Number of Bacteria Isolated**
TH (1.664021°, 103.579747°)	**JKI060**	**1170**	TH^{S1}	3	5	**8**
			TH2^{S2}	5	3	**8**
TS (1.606228°, 103.637544°)	**JKI052**	**945**	TS^{S1}	3	2	**5**
			TS2^{S2}	5	6	**11**
BP (1.1.639545°, 103.625330°)	**JKI065**	**995**	BP^{S1}	4	5	**9**
			BP2^{S2}	4	5	**9**

Antibiotic Resistance of Culturable Isolate(s) Towards Antibiotics Ciprofloxacin, Chloramphenicol, Gentamicin, Tetracycline and Sulfamethozaxole Respectively

Antibiotic susceptibility tests provide valuable insights into the antibiotic resistance of influent and effluent samples from three selected wastewater treatment plants (Figure 1) for the first sampling (S1) and second sampling (S2). The resistance was evaluated based on resistance towards five commonly used antibiotics. The results show consistent high resistance to sulfamethoxazole from both influent and effluent for S1, which is as high as 80% to 100% resistance. Meanwhile, bacterial isolates from S2 revealed high resistance to sulfamethoxazole, with 60% resistance in influent samples and 50% resistance in effluent samples. Bacteria highly resistant to certain antibiotics may be influenced by environments with high antibiotic contamination. Research by Jendrzejewska and Karwowska [24] on laboratory-scale simulation of STP laboratory-scale proves the detection of microbial resistance along with the increase in antibiotic concentrations added. The same study on antibiotics detection by Chen and fellow researchers in the same conventional WWTPs in Johor, Malaysia, revealed that the sulfamethoxazole's concentration detected in influent is the highest (1090 ± 1000 ng/l) and remained the highest in the final effluent samples with a concentration of 398.1 ± 443.3 ng/L in comparison to other antibiotics [12]. The persistently high and distinctively resistant phenotypes from influent to effluent from both samplings imply incomplete removal of multi-resistant bacteria and possible enrichment of these populations during treatment.

From the five antibiotics tested for bacterial resistance for S2, the resistance percentage towards tetracycline is around 40% from influent in one of the locations (TH2), while a range of 20-66.67% of tetracycline resistance for effluent samples BP2 (20%), TS2 (50%), and TH2 (66.67%). A percentage of resistance towards antibiotic ciprofloxacin (16.67-20%) and towards antibiotics gentamicin (20-33.33%) is also observed for bacterial isolates from all locations in influent and effluent samples in S2. Meanwhile, all bacteria in this study exhibit 0% bacterial resistance to Chloramphenicol from both influent and effluent for isolates from S2, making them the least resistant to Chloramphenicol for both influent and effluent in S2 tested in this study. Some research findings on AST of *Staphylococcus sp.* isolated from STP environment in Poland interestingly reveal that more than 50% of this species portray resistance to a different percentage towards other antibiotics, such as penicillin, erythromycin, rifampicin, tetracycline, gentamicin, cefoxitin, and levofloxacin, but are completely susceptible to chloramphenicol [21, 25]. The findings from this study show variable reduction percentages across different antibiotic classes. The fate of ARB is reported to be strongly influenced by the treatment technology and the intrinsic property of bacteria carrying the resistance [21]. which could result in partial reduction of certain antibiotic resistance after treatment.

The persistent and high level of resistance identified for bacterial isolates from effluents in this study correlates with findings that conventional WWTPs are inefficient at completely eliminating ARB. This includes research by Abdelgalel and fellow researchers in which bacterial resistance was tested for 92 out of 120 isolates isolated from hospitals, the community, and WWTPs. The indicator bacterium *E. coli* strains isolated from the study were tested for susceptibility against 11 antibiotics, and the findings showed 66.3% of the isolates exhibited resistance to at least one antibiotic, while multidrug-resistant isolates constituted 42.6% of resistant strains [23]. The results equally suggest that WWTPs act as reservoirs and a partial barrier for resistance, and resistant bacteria still persist after treatment, thus STPs can still contribute to the spread of resistance.

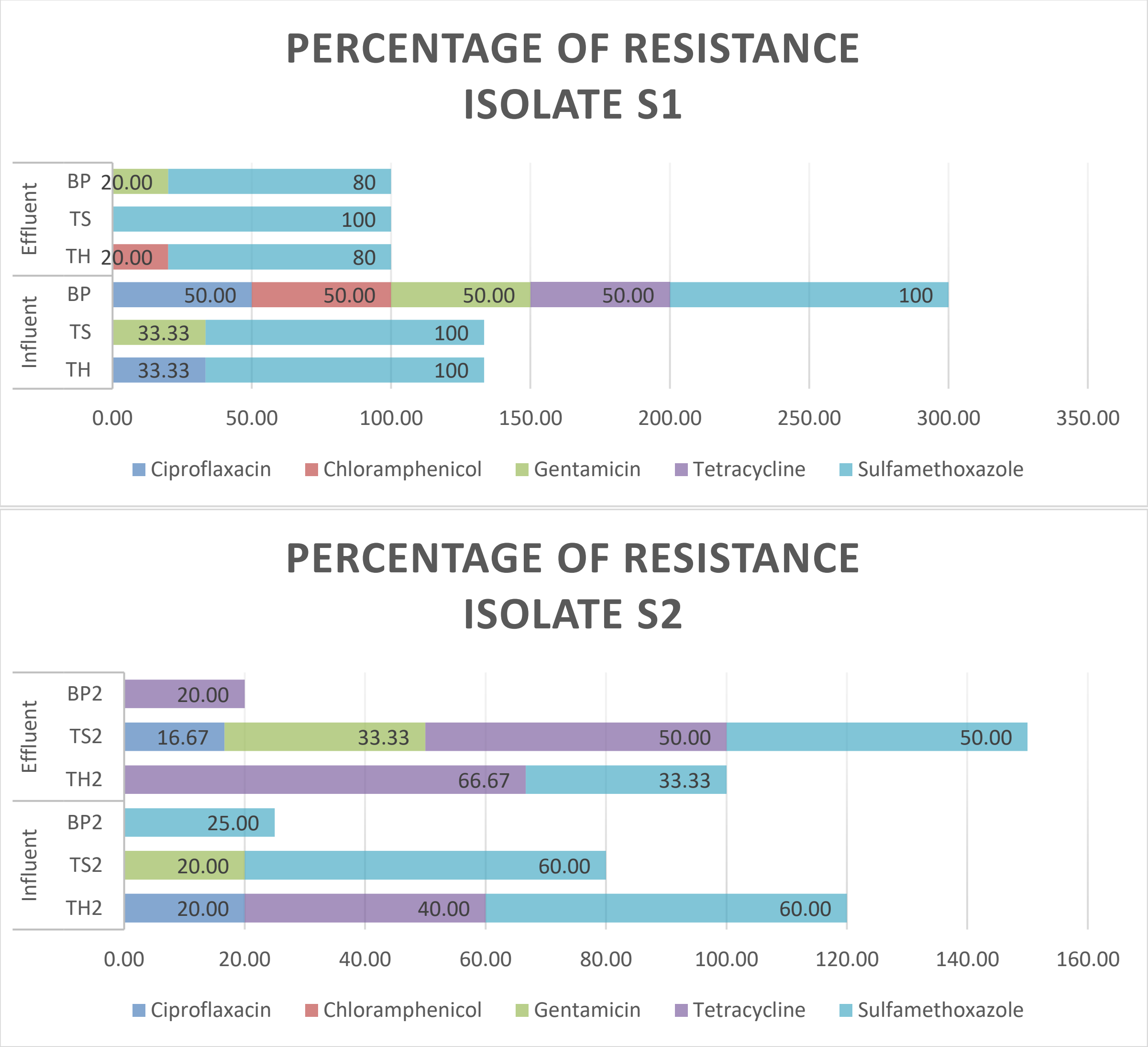

Figure 1. Percentage of resistance isolates in S1 and percentage of resistance isolates in S2

Number of Bacteria Isolates and Percentage of Isolates with MAR Index ≥ 0.2

Isolates with MAR index ≥ 0.2 for three STPs from both sampling durations are identified and shown in Figure 2. In TH, TS, and BP for S1, the influent samples contained a higher number of isolates with MAR ≥ 0.2 compared to the effluent, indicating that the incoming wastewater was a major reservoir of MDR bacteria. Although the effluent showed a reduction in the number of high isolates with MAR index ≥ 0.2, resistant bacteria were still detectable after treatment, demonstrating that conventional treatment processes did not completely eliminate antibiotic-resistant populations. Meanwhile, a similar pattern was observed in S2, but with greater persistence of high MAR isolates in effluent, particularly in TS2, where several isolates still exhibited MAR ≥ 0.2. This indicates potential seasonal or operational effects, where treatment efficiency or incoming resistance load may vary in different sampling durations.

For S2, both TH2 and TS2 locations consistently showed a higher proportion of isolates with the same or an elevated MAR index compared to BP2, suggesting that these sites may receive greater anthropogenic inputs of antibiotics. These sites may be contaminated with antibiotics that could possibly cause high resistance in bacteria [35], possibly from residential or clinical wastewater sources. The presence of MAR ≥ 0.2 isolates across all sampling points confirms that antibiotic resistance is widely distributed throughout the sewage treatment system, rather than being confined to a single inflow source. However, as the culturable isolates only represent a fraction of bacteria in WWTPs [3], these data reveal significant phenotypic susceptibility for only a portion of bacterial isolates found in WWTPs [15].

The detection of significant proportions of high MDR (MAR ≥ 0.2) isolates both before and after treatment provides important ecological and public health indicator. The MAR index analysis revealed that a considerable proportion of bacterial isolates from both influent and effluent samples exhibited MAR values ≥ 0.2, which suggest exposure to high-risk sources of antibiotic pollution [5]. The continued presence of MDR bacteria in treated effluent suggests that conventional treatment may not fully mitigate the risk of releasing resistant organisms into downstream aquatic environments, where they can interact with indigenous microbiota and enter human or animal exposure pathways [34, 44]. As wastewater systems serve as hotspots for antibiotic resistance, where resistance emerges, spreads, and enters the environment, monitoring the release of ARB in STPs is crucial for assessing potential health risks [46].

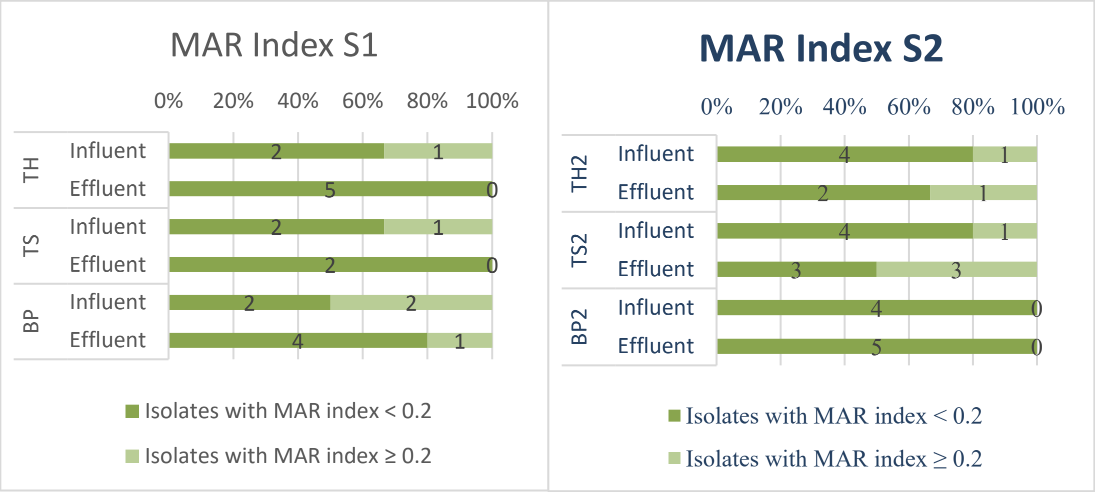

Figure 2. MAR index data for S1 (October 2018) and MAR index data for S2 (April 2019)

Culturable Multi-resistant Pathogenic Bacteria Revealed by AST and 16S rRNA Gene Sequencing (Identification of the Multi-resistant Isolate)

Eleven multi-resistant isolates in this study were screened for identification. And their resistance profile was reported for the resistance towards antibiotics ciprofloxacin, chloramphenicol, gentamicin, tetracycline, or sulfamethoxazole (Table 2). These isolates express resistance (R) to more than one class of antibiotics, which reveals the presence of multi-resistant bacteria within the sewage samples. The multi-resistant isolates were screened for 16S rRNA gene sequencing. The sequencing result of this study was deposited in GenBank, NCBI. From the sequencing data analysis, all multi-resistant bacteria isolated in this study are identified as opportunistic pathogens, in which the family of *Enterobacteriaceae* (*Citrobacter sp., Serratia sp., Enterococcus sp.,* and *Escherichia sp.*) accounts for 45.45% of the culturable multi-resistant isolates, while the *Aeromonadaceae* family and the *Pseudomonadaceae* family are 27.27% respectively.

All *Enterobacteriaceae* identified from this study are resistant to both gentamicin and sulfamethoxazole. The *Enterococcus* strain (*Enterococcus faecalis* strain TS2_E_4, a gram-positive bacterium identified from this study) is resistant to all tested antibiotics (ciprofloxacin, gentamicin, tetracycline, and sulfamethoxazole) except for chloramphenicol. Between 5% to 15% of instances of infected endocarditis are generally caused by *Enterococci*, and enterococcal endocarditis caused by these bacteria is more frequently caused by *E. faecalis* than *E. faecium* [39]. The more common *Serratia* infection reported is by *Serratia marcescens*. Although the respiratory system and wounds are usually isolated from the organism, the urinary tract is the most common source of infection [40]. Multi-resistance bacteria, especially gram-negative opportunists like *Pseudomonas sp.* and *Aeromonas sp.*, are concerning as these isolates can transfer resistance determinants to human commensals or pathogenic strains, potentially compromising clinical treatment options [47]. *Aeromonas sp.* can cause many diseases (Table 2).

Table 2. AST of multi-resistant bacteria isolated from STPs and diseases related to bacteria. CIP: ciprofloxacin; CHL: chloramphenicol; GEN: gentamicin; TET: tetracycline and SUL: sulfamethoxazole (S: Susceptible; I: Intermediate; R: Resistant)

Name of bacterial isolate(s) deposited to Genbank	Accession Number	AST (S/I/R)					Opportunistic Pathogen / Pathogen	Family	Diseases Related to Bacteria
		CIP	CHL	GEN	TET	SUL			
Pseudomonas japonica strain TH_I_1	OQ708123	R	S	S	S	R	Opportunistic	*Pseudomonadaceae*	Endocarditis, pneumonia, infections of the urinary tract, the central nervous system, wounds, the eyes, the ears, the skin, and the musculoskeletal system [38]
Aeromonas media strain TH2_I_4	OQ708124	R	S	S	R	R	Opportunistic	*Aeromonadaceae*	Food borne gastroenteritis, water-related wound infections, sepsis, peritonitis, meningitis [41]
Pseudomonas protegens strain TH2_E_1	OQ708125	S	I	S	R	R	Opportunistic	*Pseudomonadaceea*	Endocarditis, pneumonia, infections of the urinary tract, the central nervous system, wounds, the eyes, the ears, the skin, and the musculoskeletal system [38]
Citrobacter freundii strain TS_I_1	OQ708118	S	S	R	S	R	Opportunistic	*Enterocacteriaceae*	Respiratory and wound-related infections, meningitis, brain abscesses, pneumonia, diarrhea, and urinary tract infections infections [42]
Pseudomonas fragi strain TS2_I_5	OQ708119	I	S	R	S	R	Opportunistic	*Pseudomonadaceae*	Endocarditis, pneumonia, infections of the urinary tract, the central nervous system, wounds, the eyes, the ears, the skin, and the musculoskeletal system [38]
Aeromonas veronii bv. veronii strain TS2_E_2	OQ708120	I	S	I	R	R	Opportunistic	*Aeromonadaceae*	Food borne gastroenteritis, water-related wound infections, sepsis, peritonitis, meningitis [41]
Enterococcus faecalis strain TS2_E_4	OQ708121	R	I	R	R	R	Opportunistic	*Enterobacteriaceae*	Endocarditis [39]
Aeromonas caviae strain TS2_E_6	OQ708122	I	S	R	R	R	Opportunistic	*Aeromonadaceae*	Food borne gastroenteritis, water-related wound infections, sepsis, peritonitis, meningitis [41]
Serratia marcescens strain BP_I_2	OQ708115	R	R	R	R	R	Opportunistic	*Enterobacteriaceae*	Eye infections, urinary tract infections, bacteremia, pneumonia, and central nervous system infections [40]
Escherichia fergusonii strain BP_I_8	OQ708116	R	R	R	R	R	Opportunistic	*Enterobacteriaceae*	Bacteremia, diarrhea, infection in wound, urinary tract and pleural [43]
Escherichia fergusonii st rain BP_E_1	OQ708117	I	S	R	S	R	Opportunistic	*Enterobacteriaceae*	Bacteremia, diarrhea, infection in wound, urinary tract and pleural [43]

Sepsis from *Aeromonas* results from gastroenteritis or wound infection and has been reported to have a high death rate [41]. It is also reported that *Pseudomonas* infections frequently have limited treatment choices due to multidrug resistance [37]. Three *Pseudomonas sp.* identified from this study confer resistance towards ciprofloxacin and sulfamethoxazole (for *Pseudomonas japonica* strain TH_I_1), tetracycline and sulfamethoxazole (for *Pseudomonas protegens* strain TH2_E_1), and gentamicin and sulfamethoxazole (for *Pseudomonas fragi* strain TS2_I_5). Studies reported *Pseudomonas sp*. and *Aeromonas sp.* in wastewater and surface water are highly resistant to multiple antibiotics with extended-spectrum β-lactamase production. The study reveals a significant association between virulence genes and antibiotic resistance shows potential risk to communities through direct and indirect exposure to the water [47].

From the study [36] on influent and effluent samples of STPs in Johor, metagenomics analysis for bacterial community at the genus level for the most prevalent bacterial species identified in influent are *Pseudomonas* and *Janthinobacterium*, and other genera while for effluent samples are *Pseudomonas* and *Actinobacteria*, respectively. Compared to a more comprehensive metagenomics study, the isolates from this study are a subset of metagenomics, which offers a comprehensive composition of bacterial species analysis [4]. This finding reveals the multi-resistant opportunistic bacteria in STPs' influent and effluent.

Conclusions

This study reveals the prevalence of multi-resistant bacteria in STPs' influents and effluents, in which five are culturable multi-resistant opportunistic bacteria from the first sampling and six from the second sampling. Therefore, even if the richness and abundance of ARGs are reported to be significantly reduced in the WWTPs, a number of microorganisms that confers resistance to multiple antibiotics are still present in WWTPs, and thus can still be discharged into downstream habitats and affect the ecological health balance which calls for urgency in combating antibiotic resistance worldwide.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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