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The qPCR Assay for Detecting the Presence and Relative Abundance of *Pseudomonas aeruginosa* and Antibiotic Resistance Gene *aadA2* in Hospital Wastewater of National Reference Hospital

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Antimicrobial resistance is one of the top 10 global health threats. The hospital wastewater (HWW) potentially becomes the reservoir and dissemination of antibiotic resistance gene (ARG) and bacterial pathogens. In Indonesia, the protocol to monitor the ARGs form HWW has not been established. This study aimed to detect the presence and find the relative abundance of *P. aeruginosa* and *aadA2* gene from National Reference Hospital (NRH) inlet and outlet wastewater through qPCR assay. The primers used were supported by Resistomap. The study revealed that the qPCR assay was able to detect the Ct value of *P. aeruginosa* and *aadA2*. The *aadA2* gene was found in all waste water samples, meanwhile *P. aeruginosa* was only found in some of inlet samples. *aadA2* had the highest relative abundance and this gene's mobility uses plasmids and integrons that potentially enhance the acquired antimicrobial resistance (AMR) mechanism. This study implicated that qPCR assay was capable to detect pathogenic bacteria and ARG, and ARG could be released to the environment even though the wastewater samples have been proceeded in wastewater treatment plants (WWTP). The qPCR assay can be used as the method to monitor the AMR status in a hospital and the spreading potency to the environment using the HWW.

Key words: antibiotic resistance genes, antimicrobial resistance, environment, extrinsic resistance mechanism, wastewater

Resistensi antimikroba adalah salah satu dari 10 ancaman terbesar untuk kesehatan global. Air limbah rumah sakit (HWW) berpotensi menjadi reservoir dan penyebaran gen resistensi antibiotik (ARG) dan bakteri patogen. Di Indonesia, protokol untuk memantau ARG dari HWW belum ditetapkan. Penelitian ini bertujuan untuk mendeteksi keberadaan dan nilai *relative abundance* gen *P. aeruginosa* dan *aadA2* dari air limbah inlet dan outlet Rumah Sakit Rujukan Nasional (NRH) melalui uji qPCR. Primer yang digunakan dalam studi didapatkan dari Resistomap. Studi ini mengungkapkan bahwa uji qPCR mampu mendeteksi nilai Ct *P. aeruginosa* dan *aadA2*. Gen *aadA2* ditemukan pada semua sampel air limbah, sedangkan *P. aeruginosa* hanya ditemukan pada beberapa sampel inlet. *aadA2* memiliki nilai *relative abundance* tertinggi dan mobilitas gen ini menggunakan plasmid dan integron yang dapat berpotensi meningkatkan mekanisme resistensi antimikroba (AMR) dapatan. Penelitian ini mengimplikasikan bahwa uji qPCR mampu mendeteksi bakteri patogen dan ARG, serta kemungkinan dilepaskannya ARG ke lingkungan meskipun sampel air limbah telah diproses di instalasi pengolahan air limbah (IPAL) sebelumnya. Uji qPCR dapat digunakan sebagai metode untuk memantau status AMR di Rumah Sakit dan potensi penyebarannya ke lingkungan menggunakan HWW.

Kata kunci: air limbah, gen resisten antibiotik, lingkungan, mekanisme resisten ekstrinsik, resistensi antimikroba

In 2019, the World Health Organization (WHO) declared antimicrobial resistance as one of the top 10 global health threats (Holmes *et al.* 2016; WHO World Health Organization 2021). Overuse of antimicrobials is a major driver of resistance of infectious pathogens (CDC Centers for Disease Control and Prevention 2019). Antibiotic resistance in bacteria can occur because these bacteria have or overexpress the genes that encode the resistance characteristics. Mechanisms of antibiotic resistance can occur through both intrinsic and acquired mechanisms. The mechanism of intrinsic resistance is related to the genetics of bacteria that are normally present in these bacteria. The mechanism of acquired resistance is related to the horizontal transfer of antibiotic resistance genes so that resistance

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characteristic from one bacteria can be transferred to another (Holmes *et al.* 2016; Peterson and Kaur 2018).

Pseudomonas aeruginosa is a rod shaped Gram-negative bacteria with a genome length of 5.5 – 7 Mbps. It is known as an opportunistic pathogen associated with nosocomial infections and ventilator-associated pneumonia (Pachori *et al.* 2019; Pang *et al.* 2019). *P. aeruginosa* is one of the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa*, and *Enterobacter* species) which is a group of pathogenic bacteria that responsible for infections in hospitals with their ability to escape from antibiotics (Helen W Boucher, 2020). The results of the latest surveillance conducted by the European Center for Disease Prevention and Control (ECDC) showed that nearly 31% of *P. aeruginosa* isolates were resistant to at least one group of tested antibiotics (European Centre for Disease Prevention and Control. 2018). The multidrug resistance (MDR) *P. aeruginosa* arises concern because of the nature of the bacteria which is easy to adapt to various environmental conditions and can spread widely (Helen W Bouche, 2020). *P. aeruginosa* detection can be conducted by conventional bacterial culture methods, immunological assays, and molecular assays (Tang *et al.* 2017). In this study, we conducted the detection of *P. aeruginosa* through molecular assay, the qPCR, targeting the complete genome at certain region of *P. aeruginosa* as referred to Stedtfeld et al.'s study (2018).

Wastewater Treatment Plants (WWTP) are the important reservoir of antimicrobial resistance issue (Pärnänen *et al.* 2019). Study from Oliveira *et al.*(2018) reported the finding of 11% (from total 27 isolates) resistant *P. aeruginosa* isolated from WWTP Rio Para located in the City of Divinópolis, Southern Brazil. Data from Brazil showed a high profile of antibiotic resistance in *P. aeruginosa* isolated from hospital wastewater (HWW) treatment (HWWTP), the discovery of MDR *P. aeruginosa* were 85.4% in Passo Fundo, 82% in Rio de Janeiro, and 60% in Manaus. The *P. aeruginosa* isolates (15 isolates) from clinical specimens in National Reference Hospital Dr. Cipto Mangunkusumo (RSCM) from April to November 2015 were resistant to ceftazidime, ciprofloxacin, amikacin, and carbapenems thorough in vitro Vitek 2 *compact* test (Prasetyo *et al.* 2022). Amikacin is one of the aminoglycosides antibiotic group and this group is also one of drug of choice for treatment *P. aeruginosa* infection (CLSI Clinical and Laboratory Standards Institute, 2020). However, there have been no studies reporting and analyzing the profiles of pathogenic bacteria, resistance genes, and antibiotic residues contained in hospitals wastewater especially National Reference Hospital (NRH).

This research aimed to detect and find the relative abundance of *P. aeruginosa* and antibiotic resistance gene (ARG) *aadA2*, for the antimicrobial resistance monitoring study in hospital wastewater (HWW).

MATERIALS AND METHODS

The research design is a descriptive study to detect certain gene of bacteria and ARG in HWW and approved by RSCM Ethic Committee No. 21-09-0905.

Hospital Wastewater (HWW) Samples Collection. Samples were collected from Oct 25th – Nov 27th, 2021 from NRH's wastewater area inlet and outlet every 3 days. Total samples were 24 (12 samples each inlet and outlet). The inlet and outlet wastewater area were collected each 1 liter and placed in alcohol 70%-disinfected bottle. The wastewater samples were transported to laboratory and filtrated immediately using Polyethersulfone (PES) 0.22 µm membrane with 47 mm diameter. After the filtration, filter membranes were stored in -20 °C freezer until the DNA extraction step performed. The volume of filtered HWW samples were 50 mL for inlet and 100 mL for outlet. The reset of HWW samples were stored in -30 °C.

DNA Extraction. DNA extraction from filter membrane was using kit DNeasy PowerWater Kit™ (Qiagen) and following manufacture instructions. At the final step, the pellet from extraction was homogenized using elution buffer and measured for DNA concentration and purity using NanoDrop with 260/280 nm wavelength.

qPCR Assay. qPCR quantification was using SensiFAST™ SYBR ® No-ROX Kit (Bioline) with 10 µL total volume each reaction. One reaction consists of: 5 µL 2X Master mix, 0.4 µM forward primer, 0.4 µM reverse primer and 1 µL DNA (0.2 ng μL^{-1}) as template. The primers were used in this study can be seen in Table 1. The primer pair targeting the P. aeruginosa was designed to detect the species-specific complete genome of P. aeruginosa from NCBI Reference Sequence, NC_002516.2 region 1410332-1410412, "AGCGTTCGTCCTGCACAAGTTC GACGGCCTGTCCCAGGTCGAAGTGGCCGAGC GCATGGGAATCTCCCTGAGCATGGTGGA". Real-time cycling conditions included 2 min enzyme activation at 95 °C followed by 40 cycles of

Table 1 *Staphylococcus* sp. prevalence in 2017-2021

Gene	Forward Primer	Reverse Primer
P. aeruginosa PAO1, complete genome region 1410332-1410412	AGCGTTCGTCCTGCACAAGT	TCCACCATGCTCAGGGAGAT
aadA2	CAATGACATTCTTGCGGGTATC	GACCTACCAAGGCAACGCTATG
16s rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG

Table 2 DNA concentration from HWW samples

	Sampling	DNA	
		Concentration (ng μL^{-1})	Purity
Inlet	25-Oct	81.90	1.91
	28-Oct	96.80	1.95
	31-Oct	75.90	2.01
	3-Nov	142.90	1.98
	6-Nov	31.00	1.99
	9-Nov	47.00	1.95
	12-Nov	162.30	1.93
	15-Nov	123.90	1.96
	18-Nov	57.30	1.97
	21-Nov	46.80	1.90
	24-Nov	150.30	1.93
	27-Nov	57.20	1.95
Outlet	25-Oct	25.20	1.83
	28-Oct	4.80	1.22
	31-Oct	4.40	1.88
	3-Nov	1.90	1.87
	6-Nov	4.20	1.66
	9-Nov	3.20	1.76
	12-Nov	4.00	1.75
	15-Nov	3.00	1.55
	18-Nov	7.60	1.68
	21-Nov	2.00	1.38
	24-Nov	2.70	2.00
	27-Nov	3.41	2.41

denaturation at 95 °C for 5 sec and annealing 60 °C for 10 sec and elongation at 72°C for 10 sec. Each sample was tested duplo in qPCR assay.

Relative Abundance. The relative abundance was obtained from normalize each gene with 16s rRNA gene using Livak formulation

Relative abundance = $2^{-\Delta Ct}$

(ΔCt = Ct of gene – Ct of 16S rRNA gene).

RESULTS

Waste Water Samples Collection and DNA Extraction. The HWW treatment used in NRH was activated sludge with extended aeration system. The wastewater samples were collected from two spots as can be seen in HWW treatment diagram in Figure 1. The DNA concentrations from extraction process were 31.00-162.30 ng μL^{-1} and 1.90-25.20 ng μL^{-1} from inlet and outlet HWW samples, respectively (Table 2).

qPCR Assay and Relative Abundance. Based on qPCR assay (Table 3) the *P. aeruginosa* was detected at certain date in inlet wastewater samples and not detected at all in outlet wastewater samples. The *aadA2* gene was detected in all inlet and outlet samples with cycle threshold (Ct) values 17.650 to 35.285. The relative abundance value can be seen in Table 4.

Table 3 Cycle threshold (Ct) values and standard deviation (Stdev) of *P. aeruginosa* and *aadA2* in qPCR assay

	Sampling	*P. aeruginosa*		*aadA2*		*16s rRNA*	
		Ct Value	Stdev Ct	Ct Value	Stdev Ct	Ct Value	Stdev Ct
Inlet	25-Oct	36.245	0.460	19.885	0.163	17.095	0.021
	28-Oct	35.625	2.029	19.265	0.035	17.015	0.078
	31-Oct	-	-	22.170	0.339	17.400	0.057
	3-Nov	-	-	21.675	0.502	17.690	0.209
	6-Nov	-	-	22.740	0.495	18.085	0.007
	9-Nov	-	-	22.050	0.071	17.585	0.262
	12-Nov	-	-	17.650	0.368	17.380	0.028
	15-Nov	34.945	0.615	21.080	0.042	17.295	0.095
	18-Nov	-	-	17.740	0.523	17.505	0.191
	21-Nov	36.160	0.948	17.650	0.028	17.240	0.338
	24-Nov	36.335	0.021	20.925	0.163	17.205	0.021
	27-Nov	33.983	0.508	21.350	0.269	17.508	0.365
Outlet	25-Oct	-	-	22.305	0.304	19.755	0.187
	28-Oct	-	-	25.140	0.014	22.425	0.064
	31-Oct	-	-	24.335	0.163	21.725	0.148
	3-Nov	-	-	25.570	0.085	25.395	0.007
	6-Nov	-	-	32.640	0.170	25.628	0.321
	9-Nov	-	-	27.588	0.484	23.615	0.247
	12-Nov	-	-	35.285	1.549	25.640	0.354
	15-Nov	-	-	32.485	0.021	25.425	0.007
	18-Nov	-	-	23.330	0.099	21.105	0.064
	21-Nov	-	-	27.495	0.361	27.880	0.071
	24-Nov	-	-	24.330	0.085	21.035	0.078
	27-Nov	-	-	25.115	0.035	21.515	0.332

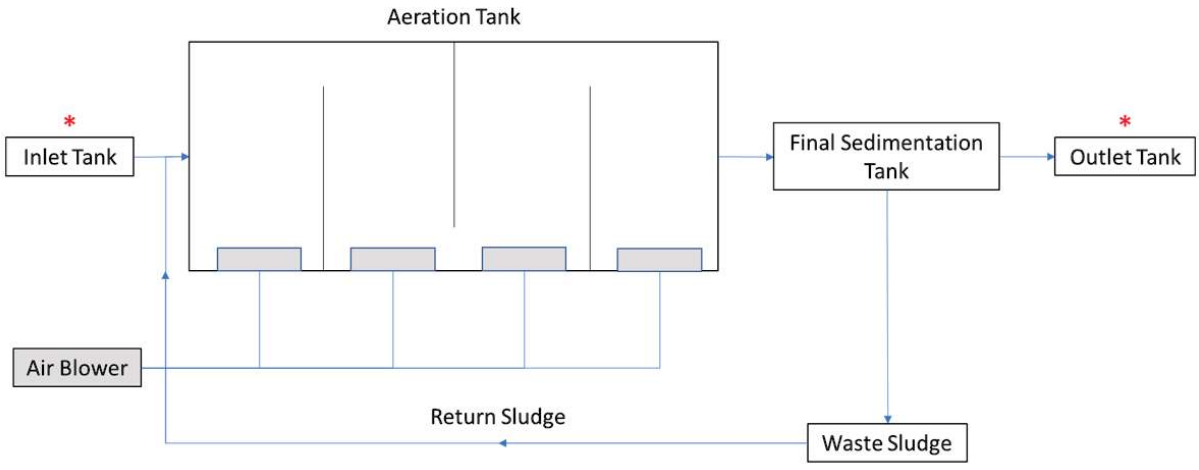

Fig 1 The diagram of NRH HWW treatment, red asterisk signs were the sampling spots.

Generally *aadA2* has higher relative abundance than *P. aeruginosa*. The relative abundance was visualized in heat map (Table 5). The darker colour shows higher value of relative abundance.

DISCUSSION

The *aadA2* gene was found constantly in wastewater samples inlet and outlet, meanwhile the *P.*

Table 4 Relative abundance *P. aeruginosa* and *aadA2* gene

	Sampling	*P. aeruginosa*	*aadA2*
Inlet	25-Oct	0.000002	0.144586
	28-Oct	0.000002	0.210224
	31-Oct		0.036651
	3-Nov		0.063153
	6-Nov		0.039692
	9-Nov		0.045279
	12-Nov		0.82932
	15-Nov	0.000005	0.072544
	18-Nov		0.849685
	21-Nov	0.000002	0.752623
	24-Nov	0.000002	0.075887
	27-Nov	0.000011	0.06971
Outlet	25-Oct		0.170755
	28-Oct		0.152301
	31-Oct		0.163799
	3-Nov		0.885768
	6-Nov		0.007745
	9-Nov		0.063703
	12-Nov		0.001249
	15-Nov		0.007494
	18-Nov		0.213899
	21-Nov		1.30586
	24-Nov		0.101884
	27-Nov		0.082469

Table 5 . Heat map relative abundance *P. aeruginosa* and *aadA2* gene

	Sampling	*P. aeruginosa*	*aadA2*
Inlet	25-Oct		
	28-Oct		
	31-Oct		
	3-Nov		
	6-Nov		
	9-Nov		
	12-Nov		
	15-Nov		
	18-Nov		
	21-Nov		
	24-Nov		
	27-Nov		
Outlet	25-Oct		
	28-Oct		
	31-Oct		
	3-Nov		
	6-Nov		
	9-Nov		
	12-Nov		
	15-Nov		
	18-Nov		
	21-Nov		
	24-Nov		
	27-Nov		

Value:

0.000002	-	1.30586

aeruginosa was found only in inlet samples. This indicated that the system of wastewater treatment has not completely eliminated the ARG. The ARG in outlet has the potency to contaminate the environment when it was released. The ARGs in studies that have been reported found in HWW were *aadA25, dfrA16, dfrA5, macB, MexF, mexW, smeE, blaVEB, aadA2, blaGES, blaVIM, AAC.6...30/AAC.6...Ib., baeR, cpxA, CRP, dfrA1, emrA, qacH, tet36, ugd* (Majlander *et al.* 2021; Cai *et al.* 2021). This should be a concern because the mobility characteristic of *aadA2* gene. According to The Comprehensive Antibiotic Resistance Database (CARD) (Alcock *et al.* 2020), *aadA2* is an aminoglycoside nucleotidyltransferase gene encoded by plasmids and integrons in *K. pneumoniae*, *Salmonella* spp., *Corynebacterium glutamicum*, *C. freundii*, and *Aeromonas* spp. This gene expresses enzyme aminoglycosides 3'-adenyltransferase and causes the resistance to streptomycin and spectinomycin. The mobility of this gene uses plasmids and integrons that means this gene can be transferred intraspecies or interspecies (horizontally) in acquired resistance mechanism. *P. aeruginosa* is also one of bacteria that was reported carrying the *aadA2* gene. The *P. aeruginosa* isolates from patients in 3 General Hospitals in Tehran, Iran were found carrying *aadA2* gene with prevalence 47.6% (Salimizadeh *et al.* 2018).

This study shows that the qPCR assay is capable to detect the presence of potential pathogen bacteria and ARG in HWW. The method can be used as the monitoring of antimicrobial resistance (AMR) status in hospital and the prevention and controlling of AMR spreading to environment. Further study can be developed to optimize the controlling of AMR such as the pathogenic bacteria isolation from HWW and then the bacteria will be assessed phenotypically (such as antibiotic susceptibility) and genotypically (PCR followed by sequencing) so the ARGs can be confirmed to be expressed in bacteria and can be traced back to see the potency of horizontal transfer.

In conclusion, the qPCR assay can be a method for monitoring pathogen bacteria and ARGs to describe the AMR status in hospital using wastewater samples.

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