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PRELIMINARY STUDY OF ANTIBIOTIC RESISTANT *ESCHERICHIA COLI* IN HOSPITAL WASTEWATER TREATMENT PLANTS IN INDONESIA

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ABSTRACT

The widespread uses of antibiotics have encouraged antibiotic resistant bacteria to develop and wastewater treatment plants (WWTPs) are believed to be hotspots for the dissemination of such bacteria. This research was conducted to ascertain the effect of WWTPs at a Jakarta public hospital on the prevalance of antibiotic resistant *E. coli* in three antibiotics, namely Meropenem, Ciprofloxacin and Cefixime, using the Kirby Bauer method. WWTPs apply activated sludge, polystyrene filtration, and chlorination to treat hospital wastewater. Raw wastewater was found to contain 4.6×10^4 CFU *E. coli* with the percentage of antibiotic-resistant *E. coli* in Meropenem of 3.8%, in Ciprofloxacin of 53.8%, and in Cefixime of 56.3%, while treated wastewater contained antibiotic resistant *E. coli* in Meropenem at the level of 20%, in Ciprofloxacin of 60%, and in Cefixime of 80% for 1.3×10^3 CFU *E. coli*. Hospital WWTPs increased the percentage of antibiotic-resistant *E. coli*. The *E. coli* becoming resistant to Meropenem, the Carbapenem class antibiotic known for its effectiveness in dealing with antibiotic-resistant bacteria.

Keywords: Antibiotics; *Escherichia coli*; Kirby Bauer; Resistant bacteria; Wastewater

1. INTRODUCTION

As more and more people move to the city, one inevitable consequence that needs to be faced is crowded living conditions with inadequate facilities and job opportunities, forcing people to live in poverty. Poverty brings problems with sanitation, malnutrition and infectious diseases, amongst others. According to the World Health Organization (WHO) and the United Nation Children's Fund (UNICEF), 700 million people have no access to drinking water (the majority in Africa), 200 million people face sanitation problems, and 1 billion people defecate in the open (WHO & UNICEF, 2014).

In 2017, the Indonesian population has reached more than 260 million people, many of whom are concentrated in the capital Jakarta (World Population Review, 2018), which had a population of 10 million people and a density of approximately 15,000 people per km^2. This dense population means the city faces problems similar ones faced by other populous cities, especially poverty, sanitation, access to drinking water, and infectious diseases (DKI Jakarta Bureau of Statistics, 2014).

Antibiotics are the key to treat diseases caused by bacteria in most countries, including Indonesia. A survey conducted by the Parent Care Foundation (Yayasan Orang Tua Peduli) revealed that in Indonesia 86.4% of cases of dengue fever and 74.1% of diarrhea were treated

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with antibiotics, even though such diseases can sometimes be treated just by resting or consuming vitamin C (Yayasan Lembaga Konsumen Indonesia, 2014). Antibiotics are used not only in medical treatment, but also in agriculture to protect high value crops from bacteria, in livestock to prevent diseases and to stimulate their growth, and in fisheries to prevent bacterial contamination (Kümmerer, 2009). From 2000 to 2010, global antibiotic consumption increased by 36%, from 54 billion to 73 billion pills (Van Boeckel et al., 2014)

These numbers show that the widespread use of antibiotics has reached a critical point. It is the major cause of the emergence of antibiotic resistant bacteria (ARB). The former health minister of Indonesia stated that at least 12,209 cases of ARB have been found, which is estimated to increase to at least 6,935 new cases annually in the country (Suara Pembaharuan, 2011).

Antibiotic resistance is defined as the ability of bacteria to grow in certain concentrations of antibiotics which are designated to inhibit such growth. The minimum concentration of antibiotic to inhibit bacterial growth is defined as Minimum Inhibitory Concentration (MIC), which is determined by scientists by various experiments. Bacteria are considered to be resistant when the antibiotic concentration needed to inhibit their growth exceeds the MIC (Drlica & Perlin, 2011).

Scientists are concerned that the growth of ARB will return us to the age before antibiotics had been discovered, when thousands of people died just because of small infections. Hence, it is very important to study the hotspots where ARB emerge, so that effective action can be taken to tackle the problem. One of these hotspots is wastewater treatment plants (WWTPs) (Rizzo et al., 2013).

A large amount of antibiotic residual is excreted from the human body. It is estimated that at least 70% of total antibiotics consumed are excreted without undergoing any changes (Kümmerer, 2009). The excreted antibiotics, having been reduced by the human metabolism, have a no-kill concentration, known as sub-inhibitor concentration. These antibiotics are mixed intensely with pathogenic bacteria in wastewater. The long journey begins from the wastewater collection system to the WWTPs. In WWTPs, more intense contact takes place: in no-kill concentration, bacteria absorb the antibiotics and begin to adapt to them, ultimately developing antibiotic resistant (Rizzo et al., 2013). At the final stage of the process, most WWTPs apply chlorination to ensure that all the microorganisms and viruses are destroyed. Seen as the key process in preventing the dissemination of ARB, many researchers have, however, found this process to have low affectivity in dealing with the problem (Everage et al., 2014).

Eventually, the treated wastewater, which still contains partially changed antibiotics, ARB and Antibiotic Resistant Gen (ARG) from destroyed ARB, is discharged into a receiving water body. The partially-changed antibiotic will induce mutation, and ARG will make genetic transformation, making the antibiotic susceptible to indigenous bacteria in the receiving water and becoming ARB (Everage et al., 2014).

In Europe and the United States, research in antibiotic resistant dissemination has been developing. The research objects have ranged from antibiotic concentration to bacteria gen in urban wastewater (Birošová et al., 2014; Everage et al., 2014; Paulus et al., 2019). In Indonesia, there is a lack of research concerning WWTPs as hotspots for ARB dissemination, although there have been many recorded cases of ARB (MIMS Today, 2017). Some of the reasons why some countries have fallen behind others in research include lack of human resources, unavailable measuring instruments, and dilution of wastewater due to high water consumption per capita per day (Al-Maadheed et al., 2019). Research should therefore start with WWTPs, where tons of microorganisms have close contact with large amounts of antibiotics, confirming the presence of ARB dissemination in WWTPs.

Hospitals commonly use antibiotics to treat patients for various diseases. They experience a 25% higher antibiotic concentration than domestic WWTPs (Paulus et al., 2019). As a result, the contact between antibiotics and bacteria is more likely to happen at WWTPs owned by hospitals. This research aims to conduct a preliminary study of antibiotic resistant Escherichia coli in each unit of the waste water treatment plant at one of Jakarta's public hospitals. The hospital will hereinafter be referred to as RSX for confidentiality purposes.

2. METHODS

2.1. Sampling Point

The WWTP at RSX applied four processes in treating wastewater; which was a mixture of laboratory, medical and domestic wastewater. These were an equalization tank; a Fluidized Bed Biofilm Reactor (FBBR) – activated sludge; up-flow filtration; and chlorination. To analyze the effect of each process on bacteria resistance, influent and effluent from each process were collected and four sampling points were selected to meet needs. The first sampling point was the equalization tank, for the WWTP initial influent; the second was the surface of the sedimentation tank for activated sludge effluent; the third was from the filtration effluent pipe; and the fourth sampling point was from the treated water storage as the effluent of chlorination.

Using 500 mL amber glass bottles, two bottles of sample were collected at each sampling point on each sampling day. Samples were collected from September to November 2015 during the daytime.

2.2. Sample Analysis

The collected samples were placed in a cooler box, transported to the laboratory, and then tested for Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), total *E. coli* colony, and percentage of antibiotic resistant *E. coli* in three antibiotics, Meropenem, Ciprofloxacin and Cefixime. The standards for COD and BOD testing were SNI 6989 (closed reflux and spectrophotometry) and SNI 6989.72:2009 (titrimetric) respectively. The total *E. coli* colony was tested using Total Plate Count (TPC), whiel antibiotic resistant *E. coli* was tested using the Kirby-Bauer method. The COD, BOD and total colony data were used to identify WWTP performance. A reduced concentration of COD, BOD or total colony throughout processes would indicate that they were running well, in order to justify the research as it was conducted at a well-performing WWTP.

The COD and BOD tests were performed on two out of four sampling times for each collected sampling bottle.

2.3. Antibiotic Resistant E. *coli* Test

To select *E. coli* from a variety of bacteria in the wastewater samples, they were inoculated at Brilliance *E. coli* (BEC). This has two chromogenic agents, Rose-gal and X-Glu. The function of Rose-gal is to detect β-galactosidase enzyme. Coliform bacteria will cleave Rose-gal by producing lactose enzyme and transform it into a pink-colored colony. On the other hand, *E. coli* will be distinguished from coliform, because this *E. coli* also produce glucuronidase enzyme, which is detected by X-Glu, giving the *E. coli* a purple color (Thermo Fisher Scientific, 2001).

The selected *E. coli* were grown in Tryptic Soya Agar (TSA). Before the bacteria were moved to Mueller Hinton Agar (MHA) for the antibiotic resistant test, the *E. coli* were standardized using 0.5 McFarland Standard (1.5×10^8 CFU/mL). The standardization was conducted to ensure that the bacteria inoculated in MHA had approximately the same total colony. Antibiotic discs were inserted after the *E. coli* had been inoculated in MHA. Each MHA petri dish was inserted with three different antibiotic discs, namely Meropenem, Ciprofloxacin and Cefixime. The antibiotic concentration in the Meropenem disc was 10 μg, for Ciprofloxacin it was 5 μg and for Cefixime 5 μg. The *E. coli* were then incubated at 35°C for 18–24 hours. Finally, the inhibition zones were measured using a ruler. Each of the measured zones was compared to the MIC antibiotic diameter inhibition zone to determine if the bacteria were resistant, intermediate or susceptible.

As presented in Figure 1, 240 inhibition zones were measured for each sampling time, with each of the antibiotics having 80 zones. Each bottled sample was inoculated in two BEC, and five colonies of *E. coli* in each BEC were inoculated in TSA. From TSA the colony was put into solution that was then standardized. Finally, each TSA had one MHA with three antibiotic discs in it.

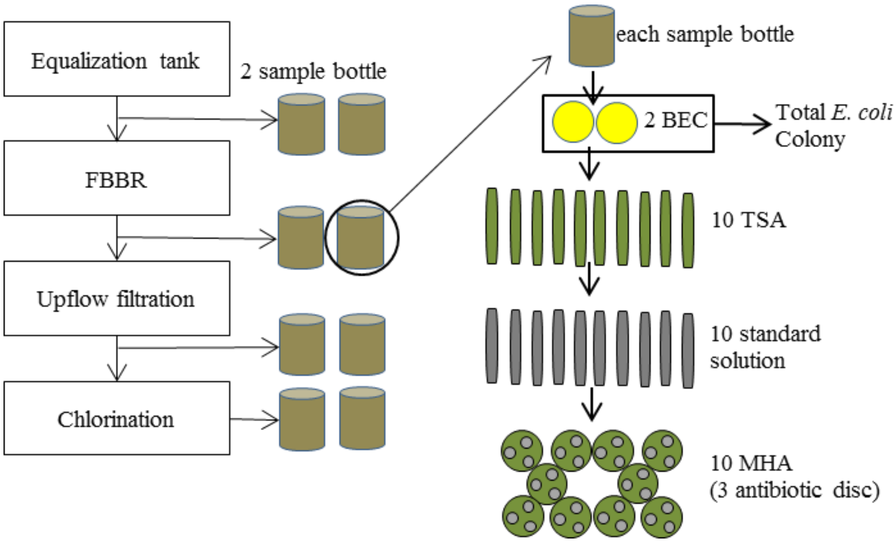

Figure 1 Antibiotic resistant *E-coli* analysis process

3. RESEARCH METHODOLOGY

3.1. WWTP Performance

Table 1 presents the average value of the COD and BOD at each sampling point on each sampling day. Each of the collected sample bottles on the sampling day was tested for both parameters. COD reduction was observed in the activated sludge unit, and removal reached more than 90% (Table 1). However, in the filtration process, the COD increased to 64 mg/L, and in the chlorination effluent, it reached 65 mg/L. This was because of the added chlorine in the chlorination process, which can be oxidized, increasing the level of detected COD (Sawyer et al., 2003). The detected BOD was proportional to the COD. In the WWTP influent and chlorination effluent, the BOD was unable to be tested. It is believed that the influent was dominated by toxic chemicals from the hospital laboratory and the chlorination effluent by chlorine. The BOD is a bioassay test, meaning nutrients for microorganism growth must be available and toxic compounds must be absent from the test sample (Sawyer et al., 2003). The COD and BOD effluent thresholds for hospital-generated wastewater are regulated by Republic of Indonesia Ministerial Decree of Environment No 58/1995 at 100 mg/L and 75 mg/L, respectively (Kementerian Lingkungan Hidup, 1995). One of the tested CODs for chlorination effluent was 65 mg/L, meaning RSX was complying with the regulation on discharging wastewater. However, on other occasion, the COD reached 351 mg/L filtration effluent, and the chlorination effluent was undetected, presumably due to high level of COD after chlorine had been added.

The *E. coli* colonies were reduced throughout the treatment process (Table 2). Beginning from the influent 4.6×10^4 CFU/mL, the detected colonies were reduced through the treatment process until sometimes were they undetected in the chlorination effluent. The WWTP performance was difficult to analyzed based on the COD and BOD data, but combined with the TPC test, it can be confirmed that the WWTP ran well overall.

Table 1 COD and BOD data

Sampling Time	Sampling Point	COD (mg/L)	BOD (mg/L)
September 29th 2015	Influent	406	-
	Activated Sludge Effluent	39	1.54
	Filtration Effluent	64	5.41
	Chlorination Effluent	65	-
November 10th 2015	Influent	1151	-
	Activated Sludge Effluent	32	8
	Filtration Effluent	351	-
	Chlorination Effluent	-	-

Table 2 *E. coli* colony data

Effluent Process	Sept 8th (CFU/mL)	Sept 29th (CFU/mL)	Oct 27th (CFU/mL)	Nov 10th (CFU/mL)	Average (CFU/mL)
Influent	2.4×10^4	2.2×10^4	7.5×10^4	6.1×10^4	$(4.6\pm2.9)\times10^4$
Activated Sludge	3.5×10^3	4.1×10^3	8.5×10^3	3.9×10^3	$(5.0\pm2.4)\times10^3$
Filtration	2.0×10^3	2.4×10^3	3.7×10^3	1.2×10^3	$(2.3\pm1.0)\times10^3$
Chlorination	1.8×10^3	0	0	7.7×10^2	$(6.4\pm8.1)\times10^2$

The constant decrease in *E. coli* colonies was not related to the fluctuating number of COD and BOD. Like this research, another study also found no relation between the presence of bacteria (in CFU/mL) and the metabolism of microorganisms (COD-BOD) in the treatment process (Ferreira da Silva et al., 2006).

3.2. Antibiotic Resistant E. *coli* at the Hospital WWTP

The colonies of *E. coli* were reduced through the treatment process. In fact, in the effluent from chlorination, no bacteria colony was found at two out of the four sampling times. However, problems appeared when the WWTP effluent still had *E. coli* and coliform in the effluent from the final treatment process. On September 8th and November 10th 2015, the effluent was found to have 1.8×10^3 CFU/mL and 7.7×10^2 CFU/mL of *E. coli* respectively.

The detected *E. coli* was tested for antibiotic resistance, and the test showed that the percentage of antibiotic-resistant *E. coli* increased after the treatment process at the RSX WWTP. Table 3 shows the percentage increase in antibiotic-resistant *E. coli* in the influent and effluent of the hospital WWTP.

Table 3 Percentage increase in antibiotic resistance before and after the treatment process

% Resistance	September 8th 2015			November 10th 2015		
	MEM	CIP	CFM	MEM	CIP	CFM
Influent	0	35	25	5	55	70
Effluent	25	50	75	15	70	85
% Increase	25	15	50	10	15	15

MEM, CIP and CFM are used as abbreviations for Meropenem, Ciprofloxacin and Cefixime, respectively. Other studies support this research, having found that the WWTP process increases the percentage of ARB (Ferreira da Silva et al., 2006; Ferreira da Silva et al., 2007; Kim et al., 2014; Guo et al., 2015).

From the overall observed data, there was a high percentage of antibiotic resistant *E. coli* to Cefixime and Ciprofloxacin. Birošová et al. (2014) also found the same result from their research conducted in Slovakia's domestic WWTP using agar diffusion method. They found that the percentage of ARB to Ciprofloxacin could reach 74%, with an average above 50%.

Other research in the northern part of Portugal found a low percentage of antibiotic resistant *Escherichia spp* to Ciprofloxacin (Ferreira da Silva et al., 2007); *Escherichia spp* is a genus of *E. coli* species. The different results could have been caused by the concentration of antibiotics in the wastewater (Kim et al., 2014) and the different treatment process applied (Bouki et al., 2013; Rizzo et al., 2013).

Research in Cheongwon's domestic WWTP in Chungbuk, Korea, provided a very clear explanation of the effect of antibiotic concentration (Kim et al., 2014). It found that antibiotics help genetic transfer in sub-inhibitor concentrations (10–100 ppb), but that they kill cells at a concentration of more than 100 ppb. It is believed that the concentration of antibiotics at the RSX WWTP was different to that in the North Portuguese WWTP. Besides, the treatment process applied and the source of the wastewater were different.

The percentage of antibiotic-resistant *E. coli* to Meropenem was observed to be less than 10% (Figure 2). Meropenem is a β-lactam class of the Carbapenem sub-category. Carbapenem is a special antibiotic designed to fight ARB, one of which is antibiotic-resistant *E. coli*. Unlike other β-lactam antibiotics, Carbapenem can resist the β-lactamase which is used by ARB to fight such an antibiotic. Unfortunately, another mechanism was developed by the bacteria to resist Carbapenem (Papp-Wallace et al., 2011) by producing Carbapenemase enzyme. The first resistance was found in 2008 (Drlica & Perlin, 2011).

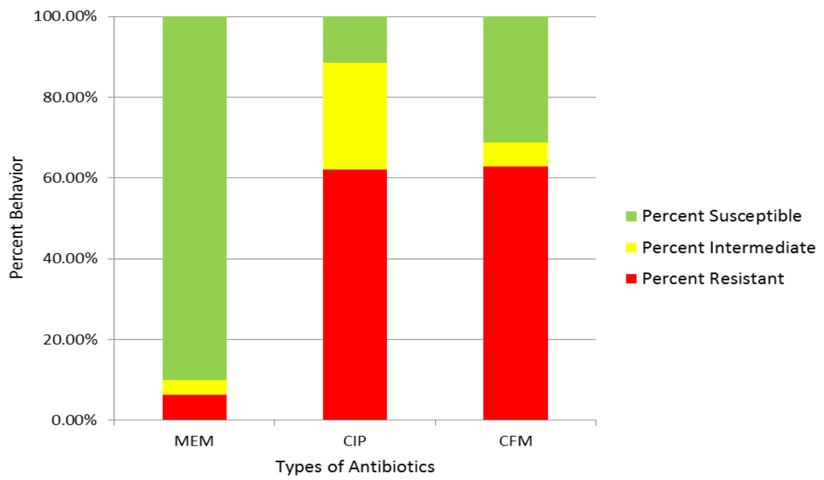

Figure 2 Percent behavior of *E. coli* after being exposed to Meropenem, Ciprofloxacin, and Cefixime

This research shows that the occurrence of Meropenem resistant bacteria are growing in Indonesia. The discharge of wastewater containing Meropenem resistant *E. coli* will induce gene transfer to indigenous bacteria, building their resistance to Meropenem (Kim et al., 2007; Szczepanowski et al., 2009; Novo et al., 2013; Kim et al., 2014; Guo et al., 2015; Tahrani et al., 2015).

Since this study only focused on *E. coli*, only a small proportion of ARB was covered. Guo et al. (2015) found that after going through the WWTP process, ARB become more diverse. Tahrani et al. (2015) found that even in treated wastewater from pharmaceutical WWTP, some pathogens such as Pseudomonas, Acinetobacter, Morganella, Shewanella, Delfia and Exiguobacterium were detected (Tahrani et al., 2015). The interesting part of their research is that the isolated bacteria in a bottle containing 3% antibiotic could survive for three weeks, meaning that the bacteria harnessed the organic carbon in the antibiotic for its growth. This provides the opportunity to harness bacteria to degrade antibiotic traces and to prevent dissemination of the antibiotic, which may solve the ARB problem.

3.3. Antibiotic Resistant *E. coli* in Activated Sludge

In activated sludge, the percentages of antibiotic resistant *E. coli* were 1.4%, 59.7% and 65.3% for the antibiotics Meropenem, Ciprofloxacin and Cefixime respectively. Table 4 shows the percentage of antibiotic-resistant *E. coli* in the total *E. coli* in each unit of the treatment process. The numbers in parentheses show the percentage of intermediate bacteria, the bacteria which will grow to become ARB (Ferreira da Silva et al., 2006; Ferreira da Silva et al., 2007).

The TPC data show that the activated sludge process was able to remove *E. coli* from the wastewater stream, but the Kirby Bauer data show that the percentage resistance of *E. coli* also increased toward Ciprofloxacin and Cefixime. Parallel to this research, many other studies have also found that although activated sludge is able to remove bacteria, the percentage of ARB continues to increase (Ferreira da Silva et al., 2006; Ferreira da Silva et al., 2007).

A further study by Gou et al. (2015), who simulated the activated sludge process on a laboratory scale to understand the resistant profile to Erythromycin, found that the resistant profile increased after completing the activated sludge process (Guo et al., 2015). Their designed bioreactor had eight hours' retention time. They stated that the heterogeneous environment in activated sludge (diverse bacteria and nutrients inside) encouraged antibiotic-resistant genes to be transferred to new bacteria, resulting in an increased percentage of ARB within a short time. To confirm this hypothesis, they also analyzed the diversity of the bacteria, and found that the range became wider. Other researches conducted on bioreactors agree that the rich condition of antibiotics, bacteria and nutrients encourages the formation of ARB (Novo et al., 2013; Szczepanowski et al., 2009).

Table 4 Percentage antibiotic-resistant (intermediate) *E. coli* in each treatment process

% Resistance (*intermediate*)	MEM	CIP	CFM
Influent	3.8 (3.8)	53.8 (35)	56.3 (6.3)
Activated Sludge Effluent	1.4 (1.4)	59.7 (26.4)	65.3 (5.6)
Filtration Effluent	6.3 (3.8)	73.8 (15)	58.8 (3.8)
Chlorination Effluent	20 (7.5)	60 (32.5)	80 (10)

3.4. Antibiotic Resistant *E. coli* in Filtration

In the filtration process, the average percentages of antibiotic-resistant *E. coli* were 6.3%, 73.8%, and 58.8% to Meropenem, Ciprofloxacin and Cefixime respectively. Comparing to the data from the activated sludge effluent, the filtration process had no consistent effect on two of the antibiotics, Ciprofloxacin and Cefixime. Similarly, research conducted on the Eriskirch WWTP in Germany using diffuse agar found that the gravel filtration process consistently decreased the percentage of antibiotic resistant *E. coli* to profloxacin and Sulfamethoxazole, but not Erythromycin and Amphicilin (Lüddeke et al., 2015). Moreover, they found that sand or gravel filtration effectively reduced the percentage of ARB, while a charcoal medium increased its percentage.

Research conducted on Shanghai Water Treatment Plant (WTP) found that activated carbon increased the percentage of ARB (Bai et al., 2015). The research explained that at first the ARB formed a biofilm, leaving the wastewater stream. After a short time, the ARB biofilm encouraged ARG transfer to the bacteria in the wastewater stream. Research in a remote part of Canada examined the performance of filtration in removing nutrients, organic micro pollutants, antibiotics and ARG (Anderson et al., 2015). Their study mentioned that a filtering medium made of grass, soil, sand and gravel could effectively remove nutrient and micro pollutants, but had no effect on antibiotics. The ARG stuck to the filter medium and encouraged gene transfer after a long period of use. The backwash can also spread ARG to the environment.

3.5. Antibiotic Resistant *E. coli* in Chlorination

The average percentages of antibiotic-resistant *E. coli* to Meropenem, Ciprofloxacin and Cefixime in the effluent from chlorination or the final WWTP effluent were 20%, 60% and 80%, respectively. Different to other treatments, chlorination is the prime disinfection process in removing bacteria (Everage et al., 2014). However, unfortunately the chlorination in this WWTP, which utilizes Trichloroisocyanuric acid (TCCA), increases the percentage of antibiotic resistant *E. coli*. in Meropenem and Ciprofloxacin, with the percentages of intermediate resistance and resistance clearly increasing after the chlorination process.

Research on a Shanghai WTP, which used chloramine in the disinfection process, found that Chloramine increased the percentage of ARB (Bai et al., 2015). According to the research, there is a possibility that ARB have more perfect structure body, making them become more resistant to oxidizers, disinfection and antibiotics than susceptible bacteria. Another research on a Nanjing WTP, which used chlorination, found that the process increased the percentage of ARB and ARG (Shi et al., 2012).

As disinfection is the prime process for removing bacteria, many previous studies were conducted to find the effective concentration to remove ARB. One study found that the effective concentration of sodium hypochlorite to remove 90% ARB was 30 mg/L, with the highest removal rate occurring between 5 to 30 min contact time, and subsequently the removal rate was decreased (Zhang et al., 2015). In addition, it was also stated that the concentration of nitrogen ammonia hinders the removal process. Another study comparing the effective concentration of disinfectants to remove ARB and ARG found the moist effective concentration of chlorine and ozone to be 30 mg/L and 3 mg/L, respectively (Oh et al., 2014).

Another study which aimed to find the effective CT (concentration and contact time) found that 50 mg Cl_2 min/L was effective to remove all types of ARB (Huang et al., 2011). However, after comparing a low concentration (2 mg/L Chlorine, 25 min) and high concentration (25 mg/L Chlorine, 2 min), they concluded that high removal efficiency depended on a high concentration of around 25 mg/L.

The RSX WWTP applied 10 mg/L chlorine with 15 minute contact time. Compared to other studies, this small concentration might be a problem; therefore, further research needs to be conducted. The concentration of ammonia in clean water is different to wastewater. Feces containing a high concentration of protein consists of an N cluster, which cannot be assimilated by the human body, with heterotrophic bacteria changing it to ammonia (Sawyer et al., 2003). A high concentration of ammonia can hinder the ARB removal process (Zhang et al., 2015).

4. CONCLUSION

The effect of hospital wastewater treatment plants on antibiotic resistant *E. coli* depends on the unit's process. Activated sludge increased by 4.17% the average of antibiotic-resistant *E. coli* due to its rich environment, which contains a wide variety of bacteria and nutrient. The filtration effect on antibiotic resistant *E. coli* depends on the filtration medium and the type of antibiotic.

In the filtration process of this research, the average antibiotic resistant *E. coli* increase is 4.17%, while on average chlorination increased antibiotic resistant *E. coli* by 7.03%. Antibiotic resistant bacteria have a more perfect structure body, making them able to withstand disinfectants better than susceptible bacteria. Overall, the process in the hospital WWTP increased antibiotic-resistant *E. coli* by around 20%.

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