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Antibiotics resistant *Escherichia coli* from hospital liquid waste

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Abstract

The emergence of antimicrobial-resistant bacteria is a major challenges in hospital waste water disposal. The present study aimed to investigate the presence of antibiotics and chlorine resistant *E.coli* in hospital liquid waste in Bogor, Indonesia. The samples were obtained from influent, chlorination tank and effluent points of waste treatment installation. The steps of the research were enumeration of bacterial population in *Nutrient Agar* (NA)and *Eosin Methylen Blue Agar* (EMBA) medium, hemolytic assay test, biochemical tests, antimicrobials test and diversities of 16S rRNA sequences analysis. The result showed the highest number of total bacterial population were at chlorination point 2.31x 10^5cfu/ml, followed by influent 1.78 x 10^5 cfu/ml, and effluent 1.9 x10^3cfu/ml. Due to hemolytic test for 30 isolates, there were 2 isolates showed beta-hemolytic sign, 4 isolates with alfa-hemolytic sign and 24 isolates showed gamma-hemolytic sign. There were a diversity resistance pattern to antimicrobial agents of *E. coli* isolates. The 16S rRNA analysis result showed that all of four tested isolates genetically closed to *E.coli*. The results of the present study gives an evidence aboutemergence possibility of multiantimicrobials resistant bacteria from insufficient disinfection method in hospital liquid waste.

Keywords: *Escherichia coli*, antibiotics resistant, hospital liquid waste

INTRODUCTION

An increasing of numbers and types of infectious diseases treated in hospital and healthcare facilities has become a major challenges in safe disposal of clinical waste in the 21st century.About ten percent of hospital generated waste is infectious, which can be hazardous to the public [1]. Empirical Study by shown that clinical liquid waste contains large numbers of microorganisms that can be detrimental to humans who come into contact with it [2]. Sharpe (2003) supposed that hospital wastewater can be risky to public health and environment as it may content pathogenic bacteria [3]. Hospital waste effluents may carry pathogenic drug-resistant bacteria. drug-resistant bacteria can spread to the environment through hospital waste water [4].

Generally, most of an effort to reduce microbial contaminant in hospital liquid waste is by using chemical treatment methods. Chlorine and its derivatives such as chlorine dioxide and sodium hypochlorite are the most widely used disinfectants to control bacterial contaminants [1]. However, there is a concern about emergence of chlorine resistant bacteria due to inefectivity of liquid waste treatment with the result unsuccesful bacterial contaminant elimination. There were some reports about unsuccesfull bacterial contaminant elimination in hospital liquid waste. Sharma *et al.* (2010) showed the appearance of antibiotics resistant bacteria in hospital liquid waste in Nepal. The bacterial isolates exhibited resistance to *Penicillin*, *Cephalosporin*, *Cotrimoxazole*, *Gentamycin*, *Quinolone* [5]. Reported assesment result of activated sludge treatment plant in Taiwan showed total bacterial population 8.1x10^7 cfu/ gr^{-1} (sludge dry weight), 4x10^6 coliform totals, 3.6x10^5fecal coliform, 1.6x10^5 fecal Streptococci, 2.2x10^5*Pseudomonas aeruginosa* and 5.5x10^4*Salmonella* spp. *Salmonella* spp. was found in 37% of sludge samples from hospital waste treatment installation[6].

The most common member of hospital waste contaminating group is *E.coli* (fecal coliform), which has been approved as a fecal pollution indicator [7]. *E. coli* is an important zoonotic pathogen. The presence of *E.coli* reveals fecal contamination to water environment [9]. *Escherichia coli* alsoknownas a common inhabitant of the intestinal tract of humans and animals and the most common cause of nosocomial- and community-acquired infections [8]. *E.coli* can transfer antibiotic-resistant genes to diverse microorganisms when exposed to antimicrobials [10]. Furthermore, inconsistency in the chlorination method is an insufficient disinfection method and it might enhance the growth of antibiotic-resistant bacteria [11].

The present study aimed to investigate the presence of antibiotics and chlorine resistant *E.coli* in hospital liquid waste in Bogor, Indonesia.This study also exert to evaluate the genetic similarity of bacterial isolates to understand their relationship.

MATERIAL and METHODS

Site and Sampling procedure

Sample obtained from liquid waste treatment installation in one government hospital in Bogor, Indonesia. Waste samples were obtained from three sampling points: influent, chlorination tank and effluent. About 250 ml liquid waste were taken using a dark sterile sample bottle. The bottles were brought in a cool box for further procedures in laboratory.

Enumeration and isolation bacteria

Bacterial population in hospital liquid waste samples were enumerated by viable count assay. One ml sample serially diluted 10^{-1}-10^{-8}, spreaded on plates with Nutrient

Agar (NA)and Eosine methylene Blue Agar (EMBA) medium. The plates were incubated at 37°C overnigt. The presence colony were counted in cfu/ml.

Identification isolates bacteria

The isolates on EMBA medium characterized by metalic green colonycolors. Single colony purified for further characterization. The isolates then stained to reveal their gram character. Also the identification involved hemolytic activity test to determine ability of isolates to lyses red-blood cells in Blood Agar Medium.Hemolytic activity determined by isolate's ability to lyse red blood cells on medium. Each isolate bacterium then biochemically characterized using indol test, Voger-Proskauer, methyil red, TSIA, sitrat test, H_2S production test.

Antibiotic susceptibility test

Nine different antibiotics were obtained from OXOID Company tested to the isolated bacteria. The interaction were determined according to manufacture's guidelines. The isolates susceptibility to chlorine were tested using commercial NaOCl. A sterile disk placed in bacterial lawn and 5,25% commercial NaOCl dropped in to disk. Inhibition zone were determined as antimicrobial activity but not antimicrobial effectivity.

16S rRNA analysis

DNA extraction conducted according to procerdures from Geneaid using Presto™ Mini gDNA Kit. Isolates bacteria were grown on *Nutrient Broth* (NB) at 30^0C overnight. DNA purity and consentration measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The DNA genom used for amplification of 16S rRNA gene. The 16S rRNA gene amplified using primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') dan 1387R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al.* 1998). PCR reaction volume were 25 µL which consist of 12.5µL of GoTag Green Master Mix 2X (Promega, Madison, W1, USA); 2.5 µL primer 63F and 1387R (consentration 10 pmol); 6.5 µL *Nuclease Free Water* and 1 µL DNA genom as template. PCR condition were *pre-denaturation* (95°C for 5 minutes), *annealing* (55°C for 1 minutes), *elongation* (72 °C for 1.5 minutes), dan *extension* (72°C for 10 minutes) with 30 cycles. PCR products visualised using electrophoresis on 1 % (w/v) agarose gel with voltage 80 Volt for 45 minutes. Visualisation conducted on UV transiluminator using Ethidium Bromida (EtBr). Nucleotide base sequences alignment with *GeneBank*data using BLAST-N (*Basic Aligment Search Tool-Nucleotide*) on online web of NCBI (*National Center for Biotechnology Information*). Phyllogenetic tree was constructed using MEGA 7.0 program by*Neighbour Joining* (NJ) method with bootsrap 1000x reps.

RESULTS

Enumeration of Bacterial population data showed that colony of bacteria were found in each sampling points. This data indicated disinfection treatment in hospital liquid waste was not fully succesfullto control population of bacteria (tabel 1).

The average of total colony on NA in chlorination tank was higher to influent and effluent. However, total colony on EMB in influent was higher to chlorination tank and effluent. Amount of metalic green colony on EMB in influent was also higher to to chlorination tank and effluent. This data showed that *E.coli* was sensitive to chlorination treatment. Those data also supported by percentage of metalic green colony in influent was higher to efluent and chlorination tank. There were 5.5% metalic green colony (supposed to be*E.coli)* of total colony in Influent, 0.17% in efluent and 0.02% in Chlorination tank.

Biochemical identification of green metalic colony confirmed all isolates were belong to *E.coli* characteristics(table. 2). An exceptional sign were found in isolate I21 that shown negative sign in indole test and isolate I26 and E5 that produces gas in TSIA test. According to hemolytic activity on Blood Agar on 30 isolates, there were 2 isolates exhibited beta-hemolytic activity. Most of all isolates were exhibited gamma-hemolytic activity (25 isolates) and the rest of isolates were alfa-hemolytic activity sign (3 isolates).

Table 1. Enumeration of bacterial population in hospital liquid waste in Bogor, Indonesia

Sampling site	Replicate	TC on NA	TC on EMB	MG on EMB	% MG to EMB	% MG to NA	% EMB to NA
Influent	1	$1.9x10^5$	$3.3x10^4$	$9.7 x10^3$	29.22	4.87	16.68
	2	$2x10^5$	$3.1x10^4$	$1.0 x10^4$	33.55	5.1	15.2
	3	$1.7x10^5$	$29x10^4$	$1.2 x10^4$	39.25	6.53	16.65
	means	$1.86x10^5$	$3.1x10^4$	$1.05x10^4$	34.01	5.5	16.17
Chlorination tank	1	$1.9 x10^5$	$4.2x10^2$	<100	9.52	0.02	0.18
	2	$1.78 x10^5$	$4.3 x10^2$	<100	6.98	0.01	0.19
	3	$1.67 x10^5$	$4.2 x10^2$	<100	9.52	0.02	0.18
	means	$1.78x10^5$	$4.23x10^2$	<100	9.52	0.02	0.18
Effluent	1	$1.9 x10^3$	<100	<100	44.12	0.16	0.36
	2	$1.7 x10^3$	<100	<100	43.48	0.17	0.39
	3	$2.1 x10^3$	<100	<100	57.14	0.2	0.34
	means	$1.9x10^3$	<100	<100	48.25	0.17	0.36

TC : total colony
MG : metalic green colony
EMB : eosine methylene blue
NA : nutrient agar

Table 2. Biochemical characteristics of hospital liquid waste bacterial isolates.

Isolates	MRVP	VP	Indole	Citrate	TSIA	Hemolitic Activity
I21	+	-	-	-	Butt=+, Slant=+, Gas= -, H2S= -	Alfa
I2	+	-	+	-	Butt=+, Slant=+, Gas= -, H2S= -	Beta
E6	+	-	+	-	Butt=+, Slant=+, Gas= -, H2S= -	Beta
I26	+	-	+	-	Butt=+, Slant=+, Gas= +, H2S= -	Gamma
K7	+	-	+	-	Butt=+, Slant=+, Gas= -, H2S= -	Alfa
E5	+	-	+	-	Butt=+, Slant=+, Gas= +, H2S= -	Alfa
I5	+	-	+	-	Butt=+, Slant=+, Gas= -, H2S= -	Gamma
E1	+	-	+	-	Butt=+, Slant=+, Gas= -, H2S= -	Beta
E2	+	-	+	-	Butt=+, Slant=+, Gas= -, H2S= -	Gamma
K11	+	-	+	-	Butt=+, Slant=+, Gas= -, H2S= -	Gamma

Due to antimicrobial susceptibility test on 10 isolates, allisolates were showed diverse resistance pattern to various antibiotics (tabel 3). All isolates were sensitive to Chlorampenicol and Levofloxacine. All isolates were resistant to Clindamycine and Rifampicine. Majority isolates were resistant to Erithromycine and Amoxiciline and sensitive to Ciprofloxacine and tetracycline. There were 4 isolates resistant and 6 isolates sensitive t oTrimetoprim Sulfamethoxazole. All isolates were showed inhibited zone to 5.25% chlorine treatment.

The result of 16S rRNA gene amplification using universal primers 63F and 1378R obtained 1376 bp sequences (table. 4). The 16S rRNA gene analysis using Genebank data by aligning the sequences using BLAST-N program showed 4 isolates (E1, K7, I21 and I2) belong to *E.coli* and 1 isolates (SI1) belong to *Cirobacter freundii*(non E.coli isolates as comparison). Percentage of similarity of isolate sequences to gene target 16S rRNA on Genebank database ranged 93-97%. Phyllogenetic analysis using sotftware MEGA also showed relationship of the isolates to *E. coli* (Figure. 1). This anaysis showed that there were *E.coli* strains diversity in this hospital liquid waste.

Discussion

In this research, we have found *E. coli* withhemolytic activity. A hemolytic activity on Blood Agar is one of bacterial virulence sign. Haemolysins are often regarded as one of the major virulence factors in various pathogenic bacteria[12]. So far, in in *E. coli*, there were three types of haemolysins have been identified. *E.coli* which exhibited Alfa-hemolytic activity is potential to produce exotoxin [13].

Nowadays, the emergence of antimicrobial resistant microorganism is the main concern in health and environment fields. Antimicrobial drugs treatment has increased the emergence of multidrugs resistant microorganism. The emergence of antimicrobial-resistant bacterial populations could caused by biological treatment processes at sewage due to selective elimination [14]. Furthermore, Korzeniewska *et al.* (2013) proposed that although wastewater treatment processes reduce bacteria number in the sewage, some antibiotic-resistant bacteria can remain in the sewage outflow [15].

In order to study the bacterial population in certain environment, the 16S sRNA sequence analysis is one of the most considerable method. The16S rRNA sequence based analysis is a central method to understand not only the microbial diversity within and across the group but also to identify new strains. Bacterial species have at least one

Table 3. Antibiotics and chlorine susceptibility pattern of hospital liquid waste bacterial isolates.

Antibiotics	Consentration	Interpretive Standard (mm)		Interpretive Criteria									
		Resistant	Sensitive	E1	K7	I21	I2	I18	I5	E6	I26	I2	K11
Chlorampenicol	30 µg/ml	≤12	≥18	S	S	S	S	S	S	S	S	S	S
Ciprofloxacine	15 µg/ml	≤15	≥21	S	I	S	I	I	S	S	I	S	S
Clindamycine	2 µg/ml	≤14	≥21	R	R	R	R	R	R	R	R	R	R
Levofloxacine	5 µg/ml	≤13	≥17	S	S	S	S	S	S	S	S	S	S
Erithromycine	15µg/ml	≤13	≥23	I	R	R	R	R	R	I	R	R	R
Tetracycline	30 µg/ml	≤14	≥19	S	S	I	S	I	I	S	I	I	S
Trimetoprim Sulfamethoxazole	1.25 µg/ml	≤10	≥16	S	R	S	R	R	S	S	R	S	S
Rifampicine	5 µg/ml	≤16	≥20	R	R	R	R	R	R	R	R	R	R
Amoxiciline	10µg/ml	≤12	≥18	S	R	S	R	R	S	S	R	S	R
Chlorine	5.25 %			+	+	+	+	+	+	+	+	+	+

S : Sensitive
I : Intermediate
R : Resistant
+ : Inhibited

Table 4. Result of BLAST-N 16S rRNA gene sequences of hospital liquid waste

Isolates codes	Strains	Total Base (isolate/ GeneBank)	Similarity	Accesion numbers
E1	*Escherichiacoli* strain SI-3	1280/1376	95%	AB609042.1
	*Shigellasonnei*strain T-X11B	1400/1376	95%	KJ806470.1
K7	*Escherichiacoli* RAD10	1493/1376	95%	KM870901.1
	Escherichiacoli strain 84	1409/1376	95%	MF399285.1
I21	*Escherichiacoli* strain E26-48	1426/1376	97%	KJ477000.1
	Escherichiacoli strain Y11R	1401/1376	97%	MF370887.1
I2	*Escherichiacoli* strain HMX3	1416/1376	94%	KY367385.1
	Corynebacteriumpseudotuberculosis strain XH-2	1394/1376	94%	MF772501.1
SI1	*Citrobacterfreundii* strain CAU6923	1385/1376	93%	M F429103.1
	Citrobacterfreundii ATCC 8090 = MTCC 1658	1827/1376	93%	KM515969.1

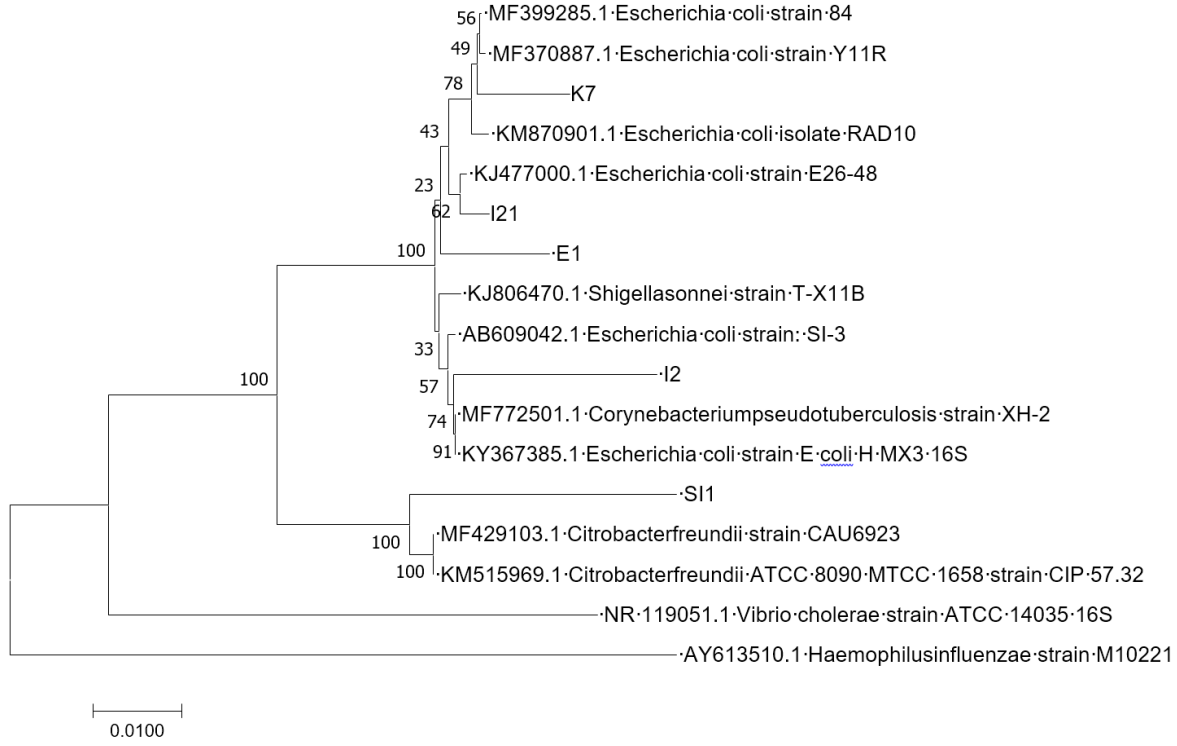

Figure 1. Evolutionary relationship of taxa using *Neighbour joining* method.

copy of the 16S rRNA gene containing highly conserved regions together with hyper variable regions, which is used for identification of new strains[16].

CONCLUSION

The present study showed a concern about efectivity of disinfection methode in controlling effort of bacteria in hospital liquid waste. Our finding gives an evidence aboutemergence possibility of multiantimicrobials resistant bacteria from insufficient disinfection method in hospital liquid waste.

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