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Profile variation of *bla* genes among non-lactose fermenting Gram-negative bacilli between clinical and environmental isolates of Dr. Soetomo Hospital, Surabaya, Indonesia

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Abstract. *Endraputra PN, Kuntaman K, Wasito EB, Shirakawa T, Raharjo D, Setyarini W. 2021. Profile variation of* bla genes *among non-lactose fermenting Gram negative bacilli between clinical and environmental isolates of Dr. Soetomo Hospital, Surabaya, Indonesia. Biodiversitas 22: 5047-5054.* Carbapenem-resistant non-fermenter Gram-negative bacilli are notorious opportunistic pathogens in hospitalized patients and hospital environments. This study explored the carbapenemase gene among non-fermenter Gram-negative bacilli from hospital wastewater and clinical isolates in Dr. Soetomo Hospital, Surabaya, Indonesia. All samples were screened on MacConkey agar with meropenem 2 µg/ml and gene detected by Multiplex PCR. All samples were screened on MacConkey agar with meropenem 2 µg/ml and gene detected by Multiplex PCR. A total of 121 isolates consisted of 76 clinical (41 carbapenem-resistant *Acinetobacter baumannii* and 35 carbapenem-resistant *Pseudomonas aeruginosa*), 45 environmental isolates (6 carbapenem-resistant *Pseudomonas aeruginosa* and 32 carbapenem-resistant *Pseudomonas* spp.), and 7 screening samples (all CRPAs). Clinical isolates carbapenemase genes were identified, $bla_{OXA\text{-}23\text{-}like}$ 21 (28%), $bla_{OXA\text{-}24\text{-}like}$ 30 (39%), $bla_{NDM\text{-}1}$ 1 (1%), and $bla_{IMP\text{-}1}$ 6 (8%) while environmental isolates were $bla_{OXA\text{-}23\text{-}like}$ 5 (13%), $bla_{OXA\text{-}24\text{-}like}$ 4 (11%), $bla_{OXA\text{-}48\text{-}like}$ 2 (5%), $bla_{NDM\text{-}1}$ 13 (34%), bla_{VIM} 12 (32%), and $bla_{IMP\text{-}1}$ 4 (11%). Rectal swab screening specimens presented $bla_{OXA\text{-}23\text{-}like}$ 3 (43%), $bla_{OXA\text{-}24\text{-}like}$ 3 (43%), and $bla_{NDM\text{-}1}$ 1 (14%). The carbapenemase gene pattern was different between clinical and environmental isolates. The $bla_{OXA\text{-}23\text{-}like}$ and $bla_{OXA\text{-}24\text{-}like}$ were most prevalent among in both clinical and wastewater, while bla_{VIM} was mostly in wastewater. The presence of carbapenem-resistant non-fermenter Gram-negative bacilli carrying carbapenemase genes in hospital effluents indicated that the community river was seeded with an antimicrobial resistance gene.

Keywords: *Acinetobacter baumannii*, carbapenemase gene, OXA, *Pseudomonas aeruginosa*, wastewater

Abbreviations: CRAB: Carbapenem resistant *Acinetobacter baumannii*, CRPA: Carbapenem resistant *Pseudomonas aeruginosa*, CROs: Carbapenem resistant organisms, MDRO: Multi-drug resistant organism, ARGs: Antimicrobial resistant genes, MGEs: Mobile genetic elements, CR-NFGNB: Carbapenem-resistant non-fermenter Gram negative bacilli

INTRODUCTION

Multi-drug resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are responsible for nosocomial infections worldwide (Kateete et al. 2016), leading to higher mortality, morbidity, longer hospital stays, and financial problems (Motbainor et al. 2020). In Southeast Asia, carbapenem-resistant *Acinetobacter baumannii* (CRAB) and carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) are commonly reported causative agents for nosocomial infections (Suwantarat and Carroll 2016), part of a rising global trend in CRAB and CRPA (Li et al. 2018; Du et al. 2019). In Indonesia, the prevalence of CRAB was higher, 50.5%, compared to 21.9% of CRPA in ICU (Karuniawati et al. 2013).

Carbapenems have been considered the most powerful drug in treating multi-drug resistant Gram-negative bacteria (Aruhomukama et al. 2019). Consequently, the increased use of carbapenem led to the global rise of carbapenemase-producing *Acinetobacter baumannii* (Du et al. 2019; Kalal et al. 2020). This enzyme is the major mechanism for carbapenem resistance in *Acinetobacter baumannii* and is divided into Ambler class A, B, and D based on amino acid sequence (Hsu et al. 2017; Beigverdi et al. 2019; Bush and Bradford 2019). The most common mechanism for CRAB is the production of a carbapenemase enzyme, predominantly oxacillinase (OXA). To date, OXA-23 still dominates the OXA group (Beigverdi et al. 2019). The presence of MBLs such as NDM, IMP, SIM, VIM has been reported in *Acinetobacter baumannii* (Abouelfetouh et al.

2019), with IMP being the most common, followed by VIM and NDM (Beigverdi et al. 2019). Hence, this rising phenomenon resulted in a critical situation in antibiotic availability against the MDR phenotype of *Acinetobacter baumannii* infections (Lukovic et al. 2020).

Pseudomonas aeruginosa presents with multiple antibiotic resistance through intrinsic, acquired, or adaptive mechanisms (Botelho et al. 2019), including its ability to acquire ß-lactamases (serine-type, MBLs, or OXAs). Even though distribution varies geographically, VIM, IMP, and NDM have been identified worldwide (Moradali et al. 2017).

The high number of antibiotic-resistant bacteria-harboring antimicrobial-resistant genes (ARGs) in the environment has been a health issue (Barancheshme and Munir 2018). Hospital environments are pooled for carbapenem-resistant organisms (CROs). Studies demonstrate that hospital wastewater hosts a variety of ARGs and resistant enteric bacteria (Marathe et al. 2019). The interplay between mobile genetic elements (MGEs), resistant bacteria, and commensals which serve as vectors for ARGs may spread into the bacterial community through horizontal gene transfer (Asfaw 2018; Marathe et al. 2019; Fouz et al. 2020). It has been hypothesized that ARGs in humans likely originate from the environment (Fouz et al. 2020).

Detection of antibiotic-resistant bacteria or ARGs by vulture or molecular based technique. As the culture-based method can recover living bacteria carrying ARGs, the molecular method may identify any ARGs from dead or alive bacteria and thus expand the probability of observing potential transfer (Rizzo et al. 2013). As previously reported, the presence of antibiotic-resistant bacteria in wastewater spotlights the possible seeding of these bacteria into the environment (Cahill et al. 2019). While significantly reducing the number of bacteria, the chlorination process potentially increases antibiotic-resistant bacteria (Rizzo et al. 2013). The study by Huang et al. (2013) demonstrated tetracycline-resistant Escherichia coli was resistant to high dose chlorination compared to susceptible strains. Following treatment, hospital wastewater will be emitted into the environment. However, it has been reported that hospital water treatment is ineffective in eliminating multi-drug resistant bacteria and or the ARGs and, therefore, significantly impacts the spread of carbapenemase producers (Dziri et al. 2020). The existence of multi-drug *Acinetobacter baumannii* outside the hospital may be a public concern since it may serve as a vector of resistance within the community environment (Anane et al. 2019).

This study aimed to analyze carbapenemase genes in carbapenem-resistant non-fermenter Gram-negative bacilli (CR-NFGNB), particularly Acinetobacter and *Pseudomonas* species, among clinical and environmental isolates.

MATERIALS AND METHODS

Bacterial isolates and identification

All clinical isolates of 41 CRABs and 35 CRPAs were collected from sputum, blood, urine, and wound specimens between mid-August to mid-November 2020 from intensive care unit, emergency department, surgical, pediatric, and medical ward excluding isolation, also urine and wound isolates from a previous study at RSUD Dr. Soetomo, Surabaya (Kuntaman et al. 2018) from those admitted to RSUD Dr. Soetomo in 2015 and 2019 respectively. These isolates were identified by BD Phoenix™ automated identification and susceptibility testing system, following the manufacturer's instructions. Carbapenem-resistant organisms were defined based on their resistance towards either meropenem or imipenem according to CLSI 2020 breakpoint for *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Table 1).

Environmental samples were collected twice a week from late August to mid-September 2020 by collecting 500 mL samples using pre-sterilized laboratory bottles from wastewater adjacent towards (surgery, internal medicine, pediatric, obstetrics and gynecology (ObGy), and intensive care unit (ICU)), hospital effluent, and inanimate objects in the ICU. Rectal swabs from ICU patients were also collected as MDRO screening. 45 mL of wastewater sample was processed by 10 minutes centrifugation at 3000xg at 4ºC and subsequently inoculating sediment onto in-house MacConkey agar by pouring 1µL of 2 µg/mL meropenem stock solution into every mL of molten MacConkey agar (final concentration of 2 µg/mL meropenem) as previously described (Zhang et al. 2020a) with modification. Rectal swabs were inoculated in BHI broth and initially incubated for 4 hours. One loop of BHI suspension was streaked on in-house MacConkey agar supplemented with 2 µg/mL meropenem and incubated 37ºC overnight. The ethical committee granted ethical approval in health research of Dr. Soetomo Hospital, Surabaya, through the following number 0065/LOE/301.4.2/ VII/2020. Informed consent was obtained from patients in ICU prior to specimen collection.

Following incubation, visible growth of non-fermenter colonies was taken for catalase and oxidase tests. DNA was extracted by boiling and submitted to multiplex PCR to identify *Acinetobacter baumannii* and *Acinetobacter* spp., with a total of 20 µL for each PCR reaction (5 µL DNA template, 0.67 µL each primer, 10 µL GoTaq Master Mix Promega), starting at 94ºC for 2 minutes for initial denaturation, then 25 cycles of denaturation at 94ºC for 1 minute, annealing at 60ºC for 30 seconds, extension at 72ºC for 1 minute, and a final extension at 72ºC for 10 minutes (Higgins et al. 2007). *Pseudomonas aeruginosa* and *Pseudomonas* spp. were identified by multiplex PCR with a total of 20 µL for each PCR reaction (5 µL DNA template, 0.5 µL each primer, 10 µL GoTaq Master Mix Promega). Multiplex PCR was started with an initial denaturation at 94ºC for 5 minutes, then 30 cycles of denaturation at 94ºC for 1 minute, annealing at 55ºC for 1 minute, extension at 72ºC for 1 minute, and a final extension at 72ºC for 10 minutes (Gholami et al. 2016). The primers for each detection are described in Table 2.

Table 1. Phenotypic identification of CRAB and CRPA from clinical isolates

Specimen type	Ward	MDRO	Meropenem	Imipenem
Sputum	Medical	CRAB	R	R
Sputum	Surgical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Wound	Surgical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Blood	Medical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Sputum	Pediatric	CRAB	R	R
Blood	Surgical	CRAB	R	R
Blood	Surgical	CRAB	R	R
Sputum	Pediatric	CRAB	R	R
Wound	Medical	CRAB	R	R
Sputum	Pediatric	CRAB	R	R
Sputum	Medical	CRPA	R	R
Sputum	Pediatric	CRAB	R	R
Blood	Pediatric	CRAB	R	R
Wound	Pediatric	CRAB	R	R
Sputum	Medical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Sputum	ED	CRAB	R	R
Sputum	Medical	CRPA	R	R
Wound	Medical	CRPA	R	R
Wound	Medical	CRAB	R	R
Blood	Pediatric	CRAB	R	R
Sputum	Medical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Blood	Surgical	CRAB	R	R
Blood	Surgical	CRAB	R	R
Wound	Surgical	CRAB	R	R
Sputum	Pediatric	CRPA	I	R
Sputum	Medical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Sputum	Surgical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Wound	Surgical	CRPA	R	R
Urine	Surgical	CRAB	R	R
Urine	Medical	CRAB	R	R
Wound	Medical	CRPA	R	R
Blood	Medical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Blood	Medical	CRAB	R	R
Wound	Surgical	CRAB	R	R
Sputum	Medical	CRAB	R	R
Sputum	Medical	CRPA	R	R
Sputum	Medical	CRPA	R	R
Blood	Medical	CRPA	R	R
Sputum	ED	CRPA	R	I
Wound	Medical	CRPA	R	R
Sputum	Medical	CRPA	R	R
Urine	Medical	CRPA	R	R
Sputum	Pediatric	CRPA	R	R
Wound	ED	CRPA	R	R
Urine	Surgical	CRPA	R	R
Urine	ED	CRPA	R	R
Urine	Medical	CRPA	R	-
Urine	Medical	CRPA	R	R
Urine	Surgical	CRPA	R	R
Urine	Surgical	CRPA	R	S
Urine	Surgical	CRPA	R	R
Urine	Medical	CRPA	S	S
Urine	Pediatric	CRPA	R	R
Urine	Medical	CRPA	R	R
Urine	Medical	CRPA	R	R
Urine	Surgical	CRPA	R	R
Urine	ED	CRPA	R	R
Wound	Pediatric	CRPA	R	R
Wound	Surgical	CRPA	R	R
Wound	Medical	CRPA	R	R
Wound	ED	CRPA	S	R
Wound	Medical	CRPA	R	R
Wound	Surgical	CRPA	I	R
Wound	Medical	CRPA	R	R

Note: Carbapenem-resistant strain was determined by the resistance against either meropenem or imipenem; ED: emergency department

Table 2. Primers for identification of *Pseudomonas aeruginosa* and *Pseudomonas* spp.; *Acinetobacter baumannii* and *Acinetobacter* spp.

Genus	Target	Sequence (5'-3')	Amplicon (bp)	Refs
Acinetobacter	*gyrB*			
	Sp2F	GTT CCT GAT CCG AAA TTC TCG	490*	Higgins et al. (2007);
	Sp4F	CAC GCC GTA AGA GTG CAT TA	294*	Higgins et al. (2010)
	Sp4R	AAC GGA GCT TGT CAG GGT TA		
Pseudomonas	*oprL*			
	F	ATG GAA ATG CTG AAA TTC GGC	504**	De Vos et al. (1997);
	R	CTT CTT CAG CTC GAC GCG ACG		Taee et al. (2014)
	oprI			
	F	ATG AAC AAC GTT CTG AAA TTC TCT GCT	249**	De Vos et al. (1997; Gholami
	R	CTT GCG GCT GGC TTT TTC CAG		et al. (2016)

Note: *: *Acinetobacter baumannii* is identified by 2 amplicons while *Acinetobacter* spp. is identified by only 1. **: *Pseudomonas aeruginosa* is identified by 2 amplicons while *Pseudomonas* spp. is identified by only 1

DNA extraction and *bla gene* detection

DNA samples from all CRAB, CRPA and CRPs were isolated by boiling method and analyzed for carbapenemase genes in two groups of multiplex PCRs. The first group was run to detect class B carbapenemase genes, with a total of 20 µL for each PCR reaction (5 µL DNA template, 0.3 each µL primer, 10 µL GoTaq Master Mix Promega) under the following conditions: initial denaturation at 95ºC for 5 minutes, continued by 30 cycles of denaturation at 95ºC for 30 seconds, annealing at 58ºC for 30 seconds, extension at 72ºC for 30 seconds, and a final extension at 72ºC for 5 minutes. The second group was performed to detect class D carbapenemase genes, with a total of 20 µL each PCR reaction (5 µL DNA template, 0.25 each µL primer, 10 µL GoTaq Master Mix Promega) under the following conditions: initial denaturation at 94ºC for 5 minutes, continued by 30 cycles of denaturation at 94ºC for 25 seconds, annealing at 52ºC for 40 seconds, extension at 72ºC for 50 seconds, and a final extension at 72ºC for 6 minutes. The primers for each detection are listed in Table 3.

Data analysis

Data were analyzed by chi-square using SPSS 25.00 with 95% confidence interval and presented into tables.

RESULTS AND DISCUSSION

In total, 121 isolates consisted of 76 clinical (41 CRAB and 35 CRPA), 38 environmental isolates (6 CRPA and 32 CRPs), and 7 screening samples (all CRPAs). We identified 58 genes of 76 clinical isolates (76.3%), 40 genes of 38 environmental isolates (105.3%), and 7 genes from 7 screening isolates (100%) which may carry ≥1 gene in a single isolate. As shown in Table 4, some non-fermenters failed to meet molecular identification and therefore were excluded.

The distribution of bla genes within the clinical specimen was dominated by sputa, with a total of 23 isolates harboring a single *bla gene*. Single *bla gene* was detected in 9 of 10 carbapenem-resistant isolates from blood specimens. We observed multiple *bla* genes in one isolate from urine and wound specimens. In urine specimens, 6 isolates with two *bla* genes and 4 isolates with single *bla gene* while in a wound, we found only one isolate with multiple *bla* genes and 8 isolates with single *bla gene* (Table 5).

These clinical isolates also showed the high number of *bla* genes recovered from medical wards among others with total of thirty-five isolates. We identified 10 $bla_{OXA\text{-}23\text{-}like}$, 21 $bla_{OXA\text{-}24\text{-}like}$, 1 $bla_{NDM\text{-}1}$, and 3 $bla_{IMP\text{-}1}$. In surgical wards, we found 4 isolates harboring two *bla* genes and 9 isolates with single *bla gene* comprised of 5 $bla_{OXA\text{-}23\text{-}like}$, 9 $bla_{OXA\text{-}24\text{-}like}$, dan 3 $bla_{IMP\text{-}1}$. On the other hand, we only observed 6 isolates with single *bla gene*, $bla_{OXA\text{-}23\text{-}like}$, from the pediatric ward (Table 6).

Thirty-nine genes from the environmental isolates were identified from wastewater comprised of 4 $bla_{OXA\text{-}23\text{-}like}$, 4 $bla_{OXA\text{-}24\text{-}like}$, 2 $bla_{OXA\text{-}48\text{-}like}$, 13 $bla_{NDM\text{-}1}$, 12 bla_{VIM}, dan 4 $bla_{IMP\text{-}1}$ and originated 12 genes from the ICU (2 $bla_{OXA\text{-}23\text{-}like}$, 2 $bla_{OXA\text{-}24\text{-}like}$, 1 $bla_{OXA\text{-}48\text{-}like}$, 2 $bla_{NDM\text{-}1}$, 4 bla_{VIM}, and 1 $bla_{IMP\text{-}1}$), 10 from ObGy (1 $bla_{OXA\text{-}24\text{-}like}$, 1 $bla_{OXA\text{-}48\text{-}like}$, 5 $bla_{NDM\text{-}1}$, and 3 bla_{VIM}), 6 from internal medicine (2 $bla_{OXA\text{-}23\text{-}like}$, 1 $bla_{NDM\text{-}1}$, 2 bla_{VIM}, and 1 $bla_{IMP\text{-}1}$), 6 from surgery (1 $bla_{OXA\text{-}24\text{-}like}$, 2 $bla_{NDM\text{-}1}$, 1 bla_{VIM}, and 1 $bla_{IMP\text{-}1}$), 4 from hospital effluent (2 $bla_{NDM\text{-}1}$ and 2 bla_{VIM}), and 1 ($bla_{NDM\text{-}1}$) from the paediatric area. Total 7 isolates from rectal swabs yielded 7 genes (3 $bla_{OXA\text{-}23\text{-}like}$, 3 $bla_{OXA\text{-}24\text{-}like}$, and 1 $bla_{NDM\text{-}1}$) and 1 gene ($bla_{OXA\text{-}23\text{-}like}$) was acquired from inanimate objects in the ICU (Table 6).

The prevalence of $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, and $bla_{NDM\text{-}1}$ from clinical CRAB was 21 (51%), 17 (41%), and 1 (2%) respectively. CRPA results were 13 (37%) for $bla_{OXA\text{-}24\text{-}like}$ and 6 (17%) for $bla_{IMP\text{-}1}$. Gene detection in environmental CRPA revealed 6 (46%) $bla_{OXA\text{-}23\text{-}like}$, 7 (54%) $bla_{OXA\text{-}24\text{-}like}$, 1 (8%) $bla_{OXA\text{-}48\text{-}like}$, 2 (15%) $bla_{NDM\text{-}1}$, and 2 (15%) $bla_{IMP\text{-}1}$. CRPs results were 2 (6%) $bla_{OXA\text{-}23\text{-}like}$, 1 (3%) $bla_{OXA\text{-}48\text{-}like}$, 12 (38%) $bla_{NDM\text{-}1}$, 12 (38%) and 2 (6%) for bla_{VIM} and $bla_{IMP\text{-}1}$ respectively (Table 7). None of the isolates harboured $bla_{OXA\text{-}58\text{-}like}$ and only two wastewater isolates were positive for $bla_{OXA\text{-}48\text{-}like}$.

Table 3. Primers for detection of carbapenemase genes

Target		Sequence (5'-3')	Amplicon (bp)	Refs
$bla_{OXA\text{-}23\text{-}like}$	F	GAT CGG ATT GGA GAA CCA GA	501	Woodford et al. (2006)
	R	ATT CTT GAC CGC ATT TCC AT		
$bla_{OXA\text{-}24\text{-}like}$	F	GGT TAG TTG GCC CCC TTA AA	246	Woodford et al. (2006)
	R	AGT TGA GCG AAA AGG GGA TT		
$bla_{OXA\text{-}48\text{-}like}$	F	TTG GTG GCA TCG ATT ATC GG	744	Poirel et al. (2012)
	R	GAG CAC TTC TTT TGT GAT GGC		
$bla_{OXA\text{-}58\text{-}like}$	F	AAG TAT TGG GGC TTG TGC TG	599	Woodford et al. (2006)
	R	CCC CTC TGC GCT CTA CAT AC		
$bla_{NDM\text{-}1}$	F	CTG AGC ACC GCA TTA GCC	754	Pfeifer et al. 2011)
	R	GGG CCG TAT GAG TGA TTG C		
bla_{VIM}	F	TGG GCC ATT CAG CCA GAT C	510	Nordmann and Poirel (2002); Nishio et al. (2004)
	R	ATG GTG TTT GGT CGC ATA TC		
$bla_{IMP\text{-}1}$	F	CTA CCG CAG CAG AGT CTT TG	587	Nishio et al. (2004)
	R	AAC CAG TTT TGC CTT ACC AT		

Table 4. Phenotypic and genotypic identification of *Acinetobacter* spp. and *Pseudomonas* spp. from environmental and MDRO screening sample

Source	Cat	Oxi	*Sp2*	*Sp4*	*oprI*	*oprL*	Result
Environmental isolates	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			-	-	Non *Pseudomonas* spp.
	+	+			-	-	Non *Pseudomonas* spp.
	+	+			+	-	CRPs
	+	+			-	-	Non *Pseudomonas* spp.
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	+	CRPA
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			-	-	Non *Pseudomonas* spp.
	+	+			-	-	Non *Pseudomonas* spp.
	+	+			-	-	Non *Pseudomonas* spp.
	+	+			+	+	CRPA
	+	+			+	+	CRPA
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			-	-	Non *Pseudomonas* spp.
	+	+			+	-	CRPs
	+	+			-	-	Non *Pseudomonas* spp.
	+	+			+	-	CRPs
	+	+			-	-	Non *Pseudomonas* spp.
	+	+			+	+	CRPA
	+	-	-	-			Non *Acinetobacter* spp.
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	+	CRPA
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			+	-	CRPs
	+	+			-	-	Non *Pseudomonas* spp.
MDRO screening sample from rectal swab specimens	+	+			+	+	CRPA
	+	+			+	+	CRPA
	+	+			-	-	Non *Pseudomonas* spp.
	+	+			+	+	CRPA
	+	+			+	+	CRPA
	+	+			+	+	CRPA
	+	+			+	+	CRPA
	+	+			+	+	CRPA

Note: Cat: Catalase, Oxi: Oxidase

Table 5. Carbapenemase gene distribution of CRAB and CRPA based on clinical specimens

Specimen	Gen *bla*			
	OXA-23	***OXA-24***	***NDM-1***	***IMP-1***
Blood	6 (67%)	2 (22%)	1 (11%)	0 (0%)
Sputum	10 (43%)	13 (57%)	0 (0%)	0 (0%)
Urine	1 (10%)	9 (90%)	0 (0%)	6 (60%)
Wound	4 (44%)	6 (67%)	0 (0%)	0 (0%)

Discussion

The predominance of *carbapenem-resistant non-lactose fermenting* Gram-negative bacilli was originated from sputum specimen compared to blood, urine, or wound. This result was consistent with the study of Cai et al. (2017) and Ssekatawa et al. (2018) which they reported the high number of CRO in respiratory tract specimens.

The prevalence of CRAB and CRPA in our hospital reached 43% and 19% in the first 6 months of 2020 (unpublished data). Clinical isolates from medical wards outnumbered others with dominant $bla_{OXA\text{-}23\text{-}like}$ in CRAB. This finding was also in agreement to Beigverdi et al. (2019). In clinical CRAB, $bla_{OXA\text{-}23\text{-}like}$ remained dominant, as found by Azimi et al. (2020), but we noticed a high number of $bla_{OXA\text{-}24\text{-}like}$ close to $bla_{OXA\text{-}23\text{-}like}$. Tafreshi et al. (2019) explained the rise of $bla_{OXA\text{-}24\text{-}like}$ was due to increased cephalosporin use. In one isolate of CRAB, the only carbapenemase gene was $bla_{NDM\text{-}1}$. Chatterjee et al. (2016) hypothesized that this gene was likely linked to Tn125 and not plasmid related.

In clinical CRPA, $bla_{OXA\text{-}24\text{-}like}$ outnumbered others. It is speculated that this gene might be disseminated horizontally since $bla_{OXA\text{-}24/40}$ is located in either plasmid or chromosome (Vrancianu et al. 2020). Moreover, 46% of clinical CRPA did not harbor any carbapenemase genes, and resistance was due to mechanisms other than enzymes. The most identified bla genes originated from the ICU area where the use of antibiotics was excessive and the remnants might have low minimum inhibitory concentration, thus potential to induce selective pressure within surrounding wastewater, generating a variety of ARGs. In our hospital, carbapenem, for instance, was administered for patients with previous MDRO infection or critically ill patients in ICU and hence likely promoted the rise of CRO through ARG transfer beyond wastewater also excreted antibiotic which might be sub-inhibitory and possibly inducing selective pressure within surrounding wastewater.

Unexpectedly, we identified CRPs carrying carbapenemase genes from hospital effluent. Those bacteria might persist during the wastewater treatment process. This finding is supported by a study in Brazil (Miranda et al. 2015) which found *Pseudomonas aeruginosa* after 5 stages of hospital wastewater treatment. Wastewater treatment is less effective for removing ARGs (Zhang et al. 2020b).

Table 6. Carbapenemase gene distribution of CRAB, CRPA, and CRPs

		Bacteria	n							
			OXA-23	***OXA-24***	***OXA-48***	***OXA-58***	***NDM-1***	***VIM***	***IMP-1***	**Total**
Clinical	Medical	CRAB	10	13	-	-	1	-	-	24
		CRPA	-	8	-	-	-	-	3	11
	Pediatric	CRAB	6	-	-	-	-	-	-	6
		CRPA	-	-	-	-	-	-	-	-
	Surgical	CRAB	5	4	-	-	-	-	-	9
		CRPA	-	5	-	-	-	-	3	8
Environment	ICU	CRPA	2	2	-	-	-	-	-	4
		CRPs	-	-	1	-	2	4	1	8
	Internal Medicine	CRPA	-	-	-	-	-	-	1	1
		CRPs	2	-	-	-	1	2	-	5
	ObGy	CRPA	-	1	1	-	1	-	-	3
		CRPs	-	-	-	-	4	3	-	7
	Pediatric	CRPA	-	-	-	-	-	-	-	-
		CRPs	-	-	-	-	1	-	-	1
	Surgical	CRPA	-	1	-	-	-	-	1	2
		CRPs	-	-	-	-	2	1	1	4
	Hospital Effluent	CRPA	-	-	-	-	-	-	-	-
		CRPs	-	-	-	-	2	2	-	4
	Inanimate Objects (ICU)	CRPA	1	-	-	-	-	-	-	1
		CRPs	-	-	-	-	-	-	-	-
MDRO Screening Sample from Rectal Swab (ICU)		CRPA	3	3	-	-	1	-	-	7
		CRPs	-	-	-	-	-	-	-	-
Total			29	37	2	0	15	-	10	

Table 7. Prevalence of carbapenemase gene among non-fermenters

***bla* Genes**	**Clinical (n=76)**	**Environmental (n=38)**	**MDRO Screening (n=7)**
Overall *bla* genes [a) b)]	51 (67%)	28 (74%)	4 (57%)
Detected *bla gene* [b)]			
OXA-23-like	21 (28%)	5 (13%)	3 (43%)
OXA-24-like	30 (39%)	4 (11%)	3 (43%)
OXA-48-like	-	2 (5%)	-
NDM-1	1 (1%)	13 (34%)	1 (14%)
VIM	-	12 (32%)	-
IMP-1	6 (8%)	4 (11%)	-

Note: [a)] Isoltaes harbouring any *bla* genes, [b)] *bla* genes per total isolates

The magnitude of *Pseudomonas* sp. in the environment, including hospital wastewater, is well understood (Spindler et al. 2012; Falodun et al. 2019). *Pseudomonas* sp. (28.2%) was found five times higher than *Acinetobacter* sp. (5.6%) from hospital wastewater in Singapore (Haller et al. 2018). We believe this dissemination of $bla_{OXA\text{-}24\text{-}like}$ was derived from ARGs in the environment. Many environmental CRPs harboured $bla_{NDM\text{-}1}$ and bla_{VIM} which might originate from *Enterobacteriaceae* since MBL genes are located in mobile genetic elements (Shams et al. 2018).

This present study did not identify any $bla_{OXA\text{-}58\text{-}like}$. This might be due to its susceptibility to carbapenem. Chen et al. (2018) identified $bla_{OXA\text{-}58\text{-}like}$ in one isolate among 23 clinical CSAB with MICs of 0.5 µg/mL and 0.25 µg/mL for imipenem and meropenem respectively. Wang et al. (2018) and Ranjbar et al. (2019) successfully recovered *Acinetobacter baumannii* but none harboured $bla_{OXA\text{-}58\text{-}like}$.

In conclusion, we found that $bla_{OXA\text{-}23\text{-}like}$ and $bla_{OXA\text{-}24\text{-}like}$ were prevalent in both clinical isolates and hospital environments while $bla_{NDM\text{-}1}$ and bla_{VIM} were only in the hospital wastewater environment. We also identified CR-NFGNB carrying carbapenemase genes in hospital water effluent, indicating that the community river was likely contaminated with ARGs and/or CROs. This alarming dissemination of a resistant bacterial community in the environment possibly constitutes a public health issue. The present study did not analyze the clonal relatedness between clinical and environmental isolates among similar *bla* genes. Due to this limitation, further research is needed to understand factors and other mechanisms that induce the rise of multi-drug resistant bacteria within hospital wastewater and the community water to prevent the spread of CRO.

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