ICU-acquired Carbapenem-non-susceptible Bacilli in Indonesia
Focus on:
Acinetobacter baumannii, Klebsiella pneumoniae and *Pseudomonas aeruginosa*

Yulia Rosa Saharman

The research described in this thesis was performed at the Department of Microbiology and Infectious Disease, Erasmus University Medical Center (Erasmus MC), Rotterdam, The Netherlands; Department of Microbiology Faculty of Medicine Universitas Indonesia, and Dr CiptoMangunkusumo Hospital, Jakarta, Indonesia.

The studies described in this thesis were financially supported by DIKTI NESSO Scholarship from The Directorate General of Higher Education of Indonesia Ministry of Education and Culture of the Republic of Indonesia and internal grants of the Department of Medical Microbiology and Infectious Disease, Erasmus MC, Rotterdam, The Netherlands

Cover and design : Yulia Rosa Saharman, Bob Adrian Saharman, Nories Khrisna Murti
Design Layout : Yulia Rosa Saharman , Chardiono Panjaitan
Print : ProefschriftMaken
ISBN : 978-94-6423-048-2

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ICU-verworven Carbapenem-niet-gevoelige Bacillen in Indonesië
Focus op:
Acinetobacter baumannii, Klebsiella pneumoniae en *Pseudomonas aeruginosa*

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

prof. R.C.M.E. Engels

and in accordance with the decision of the Doctorate Board.

The public defence shall be held on

Friday 27th November 2020 at 13.30 hrs

by

Yulia Rosa Saharman
born in Padang, Indonesia.

Erasmus University Rotterdam

PROMOTIECOMMISSIE:

Promotor	: prof.dr. H.A. Verbrugh
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Copromotoren	: Dr. J.A. Severin PhD Dr. A. Karuniawati PhD

I heartily dedicate this thesis to my family,
especially to my parents, who has provided unconditional
love and faith;
Alm Saharman Leman & Almh Gusniar Said

to my beloved husband, my soulmate; Yanfaunnas,
who fully support me and always standing beside me throughout
my journey;

to; Farras, Atika, Faris, my soul, my precious

CONTENTS

CHAPTER 1

1

General Introduction and Outline of this thesis

INTRODUCTION

This thesis addresses the increasing problem of multidrug resistant microorganisms causing infections in healthcare, especially hospital-acquired infections, in countries that are less well developed compared to most Western European and North American countries. Healthcare-associated infections constitute a sizable burden of disease, are associated with increased mortality and with increased costs of health care. Unfortunately, healthcare infections are also increasingly caused by certain species of microorganisms that display multiple resistances against commonly used antimicrobial agents.

The emergence of multidrug-resistant hospital pathogens is part of the worldwide emergence of resistance against antimicrobial agents among all microorganisms exposed to them. Antimicrobial agents as they have been and still are applied by humans in all sectors of society, including animal husbandry. This global emergence of antimicrobial resistance has reached such pandemic proportions that, in the year 2014 , it has been recognized as a special threat to mankind by the World Health Organization (WHO) and, in a special assembly on September 21st , 2016, it was declared 'the greatest and most urgent global risk' by the United Nations. Under WHO's guidance all countries are now addressing this threat.(1)

However, not all regions of the world are equally affected by this calamity, the burden of antimicrobial resistance is likely to be much higher among less well-developed countries in Asia and Africa.(2) Indonesia is one of the most populous countries belonging to the so called lower-middle income countries (LMIC) in South East Asia where antibiotic use and resistance to antibiotics are increasing rapidly (3), but where little research on the determinants of usage and of the emergence of antimicrobial resistance has taken place. When focusing on healthcare-associated infections, patients on intensive care units (ICUs) are especially vulnerable to infections acquired during their stay. As evident from our scoping review, presented and fully referenced in Chapter 2, patients in ICUs in LMIC, although little studied, may have ICU-acquired infections at rates comparable to or somewhat higher than patients in ICU's in high income countries. However, the mortality rates in LMIC ICU's are generally higher, almost twice as high as in high income countries. In addition, the spectrum of causative microorganisms and the level of multidrug resistance among them may be significantly different and higher, respectively, when compared to the pathogens causing ICU infections in high income countries.

The research presented in this thesis directly addresses the problem of ICU-acquired infections caused by multidrug-resistant pathogens in an Indonesian setting, i.e. ICUs in the national referral hospital in Jakarta, Indonesia. It aimed to provide better insight into the epidemiology of multidrug-resistant pathogens, and to explore ways to reduce the risk of acquiring such pathogens in this setting. The research was focused on the three most prevalent species of bacterial pathogens, *Acinetobacter baumanni, Pseudomonas aeruginosa* and *Klebsiella*

pneumoniae that caused the majority of serious ICU infections in this low-resource setting at the time of initiation of the studies presented here. Increasingly, clinical isolates of these three species from patients in ICUs were resistant to multiple classes of antimicrobial agents, including the last resort agents such as the carbapenem class of beta-lactam antibiotics.(4-6)

Carbapenems constitute a novel class of beta-lactam antibiotics that were discovered almost 40 years ago by Kahan et al. (JAC 1983;12(D):1-35) as a product of *Streptomyces cattleya*.(7) The first carbapenem, imipenem-cilastatin, was introduced into clinical practice only in the late eighties of the previous century, at a time that the global emergence of resistance against most classes of antibiotics was starting to become recognized.(8) Carbapenems quickly became the drug of choice in treating infections by bacteria that had become resistant to third generation cephalosporins – a much used class of beta-lactam antibiotics since their introduction in the early eighties of the previous century -. Resistance to third generation cephalosporins was due to the acquired ability of many bacteria to produce so called extended spectrum beta-lactamase (ESBL) enzymes that can degrade most beta-lactam antibiotic available at that time. In contrast, carbapenems are not degraded by ESBL enzymes. Unfortunately, but predictably, the introduction of carbapenems and their popularization led, within 10 years, to an increasing number of reports on the emergence of resistance against carbapenems, especially among Gram-negative bacilli causing healthcare-associated infection. Carbapenem resistance was found to be due to multiple mechanisms, paramount among which was the acquired ability by several species of pathogenic bacteria to produce carbapenemases, i.e. enzymes that are able to degrade carbapenem molecules. As with ESBL, many types of such carbapenemases have since been discovered over the past two decades.(9)

Collaborative studies between Indonesia and the Netherlands on this topic have been performed since the inception in 1997 of the Science Program Indonesia Netherlands (SPIN), a granting system jointly executed by the Dutch and Indonesian Academies of Sciences, KNAW and AIPI, respectively. Together with Diponegoro University in Semarang, Airlangga University in Surabaya, Leiden University Medical Centre, Radboud University Medical Center in Nijmegen, and Erasmus University Medical Center in Rotterdam, the Antimicrobial Resistance in Indonesia (AMRIN) study started in the year 2000. Little antibiotic resistance was detected among commensal Gram-negative bacilli isolated from community dwellers but many patients admitted to hospitals became colonized with Gram-negative bacilli resistant to multiple antibiotics indicating that Indonesian hospitals were important sites for the acquisition and spread of antibiotic resistance.(10) In a later study over a 4-month study period (January – April 2005) in Dr Soetomo Hospital, Surabaya, the authors found resistance to third generation cephalosporins – phenotypically due to ESBL production - in 28.5 % (115/403) of *E.coli* and in 35.7% (104/291) *K. pneumoniae* strains.(11, 12) Saharman et al. reported in 2008 on 129 ICU patients using

mechanical ventilator in the national referral hospital RS dr Cipto Mangunkusumo (RSCM) in Jakarta, that 46 of them (36%) suffered from ventilator associated pneumonia (VAP).(12) Microorganisms isolated from oropharyngeal swabs of these patients yielded *Acinetobacter anitratus* (now called *A. baumannii*) (in 23%), *Klebsiella pneumoniae* (15%), P*seudomonas aeruginosa* (13%) and methicillin-resistant *Staphylococcus aureus* (MRSA) (1,4%). Bacteria isolated from quantitative culture of bronchoscopically retrieved specimens revealed a similar distribution: *A. anitratus* (32,4%), *P. aeruginosa* (24.7%), *K. pneumoniae* (10.4 %), and *MRSA* (2.6%). The prevalence of resistance to carbapenem class antibiotics among the Gram-negative bacterial species isolated from ICU patients in RSCM was already quite high at that time, varying from 21.9 % for *P. aeruginosa* to 27.6% for Enterobacterial species and 50.5% for *A. baumannii* (13).

Aims and outline of the studies presented in this thesis.

As stated above the work presented in this thesis focused on the epidemiology of multidrug-resistant pathogens in ICUs in RSCM, and on the prevention of acquisition and spread of *A. baumannii, P. aeruginosa and K. pneumonia*, especially of isolates that were carbapenem-non-susceptible. The ultimate aim was to reduce the emergence and spread of multidrug-resistant organisms in an ICU setting with relatively low resources. More specifically, the goals and aims of the research described in this thesis were:

1. To obtain a baseline insight into the epidemiology and the phenotypic and genetic characteristics of carbapenem-non-susceptible strains of *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in a low resource ICU setting.
2. To develop an intervention - feasible to be applied in a low-resource ICU setting - that may significantly reduce the risk of acquisition and infection by carbapenem-non-susceptible *A. baumannii, P. aeruginosa* and *K. pneumoniae.*
3. To apply and determine the efficacy of the intervention (developed as specified above/ under 2) in a low resource ICU setting.

These issues and goals were addressed in ICUs of the RSCM, the national referral hospital situated in Jakarta, Indonesia, and are described in the following parts and chapters of this thesis:

Part I contains the Introduction to this thesis with a separate accompanying systematic scoping review of the literature regarding infections and antimicrobial resistance in Intensive Care Units in Lower-Middle Income countries (chapter 2).The scoping review is up-to-date, it covers literature published in high quality in the period 2005-2018, effectively including the time when most of the research presented in this thesis was performed.

Part II of the thesis contains chapters describing the molecular epidemiology of carbapenem-non-susceptible strains of *Acinetobacter baumannii* (chapter 3), *Klebsiella pneumoniae* (chapter 4) and *Pseudomonas aeruginosa* (chapter 5) in two separate ICUs in RSCM, Jakarta, Indonesia. The methods developed and applied for detailed genomic analysis of the carbapenem-non-susceptible *P. aeruginosa* isolates is presented in chapter 6.

Part III of the thesis describes the design and application of a multimodal intervention aimed to reduce the rate of acquisitions of carbapenem-non-susceptible strains of *A. baumannii, P. aeruginosa* and *K. pneumoniae* by patients admitted to two ICUs of RSCM. The effect of a multifaceted hand hygiene improvement program – part of the intervention – is presented in a separate paper (chapter 7). However, the impact of introducing the whole intervention bundle on the rate of acquisitions of carbapenem-non-susceptible strains of the three species is presented in Chapter 8. In chapter 9 we explored in more detail the effects of the intervention on the clonal composition of *P. aeruginosa* in these two ICUs.

Part IV contains a summarizing discussion of the results of the studies presented, provides a perspective on their application in clinical practice and contains suggestions for further research on the issue of preventing ICU acquired infections by multidrug resistant bacteria. It also contains the candidates curriculum vitae and her PhD research portfolio.

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CHAPTER 2

2

Systematic Scoping Review: Infections and Antimicrobial Resistance in Intensive Care Units in Lower-Middle Income Countries: A Scoping Review

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2. Department of Medical Microbiology and Infectious Diseases, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands.

Submitted

ABSTRACT

Background:

Intensive Care Units (ICUs) in Lower-Middle Income Countries (LMICs) are suspected to constitute a special risk for patients of acquiring infection due to multiple antibiotic resistant organisms. The aim of this systematic scoping review was to present the data published on ICU-acquired infections and on antimicrobial resistance observed in ICUs in LMICs over a 13-year period. A systematic scoping review was conducted according to the PRISMA extension guideline for scoping reviews and registered in the Open Science Framework.

Main body of the abstract:

Articles were sought that reported on ICU-acquired infection in LMICs between 2005 – 2018. Two reviewers parallelly reviewed 1,961 titles and abstracts retrieved from five data banks, found 274 eligible and finally included 51. Most LMICs had not produced reports in Q1 or Q2 Journals in this period, constituting a large gap in knowledge. However, from the reported evidence it is clear that the rate of ICU-acquired infections was comparable, albeit approximately 10% higher, in LMICs compared to high income countries. In contrast, ICU mortality was much higher in LMICs (33.6%) than in high income countries (<20%). Multidrug-resistant Gram-negative species, especially *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* played a much more dominant role in LMIC ICUs than in those in high income countries. However, interventions to improve this situation have been shown to be feasible and effective, even cost-effective.

Conclusions:

Compared to high income countries the burden of ICU-acquired infection is higher in LMICs, as is the level of antimicrobial resistance; the pathogen distribution is also different. However, there is evidence that interventions are feasible and may be quite effective in these settings.

The protocol was registered with Open Science Framework (https://osf.io/c8vjk).

Keywords:

Intensive care units, bacterial drug resistance, cross infection, Acinetobacter, infection control

INTRODUCTION

Approximately fifty countries of the world belong to the category of lower-middle income countries (LMIC) according to the long-standing classification by the World Bank and updated every year.(1) These LMICs share the same bracket of Gross National Income (GNI) per capita - $1,026 and $3,955 (2019) - a proxy for the level of their economic progress. This LMIC group is a quite diverse group by region, size, population, and income level, ranging from tiny nations with small populations to giants like India and Indonesia (Figure 1).

Figure 1. Global Map highlighting Lower-Middle Income Countries (blue).

LMICs are known to be affected by the worldwide pandemic of antimicrobial resistance. Patients admitted to intensive care units (ICUs) are particularly at risk of acquiring hospital-acquired infection due to multiple antibiotic resistant strains of notorious nosocomial pathogens including *Enterococcus spp., Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Escherichia coli* (a.k.a. the ESKAPE group of pathogens).(2) However, data on the occurrence and determinants of ICU-acquired infections and antimicrobial resistant pathogens from LMICs are relatively rare and published wide apart. We, therefore, present here a scoping review of the data published on the infections and antimicrobial resistance observed in ICUs in LMICs over a 13-year period and published in esteemed scientific journals. We focused on revealing which LMICs have produced relevant information in this period, and which not, what type of ICU infections were observed and at what frequencies, which species and types caused these ICU-acquired infections, and present their antibiotic resistance profile. In addition, information was sought about the role of healthcare workers (HCWs) and the ICU environment, and whether intervention studies were performed and, if so, successful in reducing (risk of) infections in these settings.

METHODS

Protocol and registration

The scoping review protocol was developed as recently recommended by PRISMA extension for scoping reviews (3-5) and registered with Open Science Framework, an international prospective register of systematic scoping reviews, on 13th December 2019 (https://osf.io/c8vjk).(3, 4)

Eligibility criteria

Any study that targeted the etiology and management of nosocomial bacterial infections in adult ICUs in LMICs, with a focus on antimicrobial resistance and interventions applied were eligible. Also, results of screening for multidrug-resistant bacterial pathogens (ESKAPE species) among humans (patients and healthcare workers) and the hospital environment were considered eligible for inclusion in this review.

The population, intervention, comparison, and outcome (PICO) framework for determining the eligibility of the studies for the primary research question is presented in Table 1.

Table 1. Inclusion and exclusion criteria for this scoping review.

Criteria	Inclusion	Exclusion
Population	Human Adult Intensive care units ICU infections, especially those acquired during ICU stay Laboratory results of screening for the presence of multidrug-resistant bacteria, especially ESKAPE species among ICU patients, healthcare workers, or the ICU environment Lower-middle income countries	Animal, Plants Children and Neonates Other hospital wards
Intervention	Preventive measures to limit nosocomial acquisition and infection of bacterial pathogens	
Comparator	Not Applicable	
Outcomes	Infection and/or acquisition Identification and susceptibility pattern of targeted pathogens (ESKAPE species) Compliance with prevention protocols (e.g. hand hygiene) Mortality Length of stay	
Language	English Language	
Study design	• Case control study • Cohort studies • Cross-sectional studies • Longitudinal studies • Modelling studies • Laboratory based studies	• Editorials • Case series reports • Conference abstracts/reports • Reviews
Quality of Journal	• Q1 or Q2 based on rank on Web of Science	• Q3 or Q4, or not ranked in Web of Science

Information sources and search

We conducted a systematic scoping review of epidemiology and management of multidrug-resistant bacteria in adult ICUs in LMICs according to the World Bank (WB) classification in 2015. As stated by the WB, for the 2015 fiscal year, lower-middle income economies were those with a GNI per capita between $1,026 and $4,035. The term country refers to any territory for which authorities report separate social or economic statistics.

The scoping review protocol was developed and registered in the Open Science Framework, as recently recommended by PRISMA extension for scoping reviews.(3, 4) A systematic scoping review is a type of evidence synthesis method aimed at mapping the range of literature that exists around a specific topic of interest and focuses the research questions by charting the existing research findings and identifying research gaps. Scoping methodology is also considered a useful approach for determining the need and value of a future primary (in-depth study) or a full systematic review.(3)

The review is restricted to papers published from January 1st 2005 until January 1st 2018, a time frame that essentially would allow all LMICs to contribute relevant data, and recent enough to still be relevant for today. OvidSP "Medline, EMBASE", Web of Science, Cochrane, Google scholar were searched. The relevant literatures were identified using the following search strings:

Embase.com

('intensive care unit'/exp OR (((('intensive care' OR 'critical care') NEXT/1 unit*) OR icu OR icus):ab,ti) AND (infection/exp OR 'antibiotic resistance'/exp OR 'infection prevention'/exp OR 'infection control'/exp OR 'vancomycin resistant Enterococcus'/de OR 'methicillin resistant Staphylococcus aureus'/de OR 'extended spectrum beta lactamase'/de OR 'carbapenemase'/de OR 'Pseudomonas aeruginosa'/exp OR 'Acinetobacter baumannii'/exp OR (infection* OR sepsis OR septic OR nosocomial* OR mrsa OR ((multidrug OR multi-drug OR resistan*) NEAR/3 (bacter*)) OR ((vancomycin OR methicillin OR carbapenem) NEAR/3 resistan*) OR vre OR mrsa OR esbl OR (antibiotic* NEAR/3 resistan*) OR 'extended spectrum beta lactamase' OR 'extended spectrum β lactamase' OR 'Pseudomonas aeruginosa' OR 'Acinetobacter baumannii'):ab,ti) AND ((('lower middle' OR 'low middle' OR 'low- and middle') NEAR/6 income NEAR/3 countr*) OR lmic OR lmics OR Armenia* OR Mongolia* OR Bhutan* OR Morocc* OR Bolivia* OR Nicaragua* OR (Cabo NEXT/1 Verde*) OR Nigeria* OR Cameroon* OR Pakistan* OR Congo* OR ('Papua New' NEXT/1 Guinea*) OR 'Cote d Ivoire' OR Paraguay* OR Djibout* OR Philippin* OR Egypt* OR Samoa* OR Salvador* OR 'Sao Tome and Principe' OR Georgia* OR Senegal* OR Ghan* OR 'Solomon Islands' OR Guatemal* OR Guyana* OR (Sri NEXT/1 Lank*) OR Hondur* OR Sudan* OR India OR Swaziland* OR Indonesia* OR Syria* OR Kiribati* OR 'Timor-Leste' OR Kosov* OR Ukrain* OR Kyrgyz* OR Uzbek* OR Lao OR laos OR Vanuatu* OR Lesotho* OR Vietnam* OR

Mauritania* OR ('West Bank' NEXT/2 Gaza) OR Micronesia* OR Yemen* OR Moldova* OR Zambia*):de,ab,ti NOT (((child/exp OR pediatrics/exp) NOT adult/exp) OR (pediatric* OR picu OR nicu OR picus OR nicus):ab,ti)

Medline (OvidSP)

(Intensive Care Units/ OR (((intensive care OR critical care) ADJ unit*) OR icu OR icus).ab,ti.) AND (exp infection/ OR exp Drug Resistance, Microbial/ OR Vancomycin-Resistant Enterococci/ OR Methicillin-Resistant Staphylococcus aureus/ OR Pseudomonas aeruginosa/ OR Acinetobacter baumannii/ OR (infection* OR sepsis OR septic OR nosocomial* OR mrsa OR ((multidrug OR multi-drug OR resistan*) ADJ3 (bacter*)) OR ((vancomycin OR methicillin OR carbapenem) ADJ3 resistan*) OR vre OR mrsa OR esbl OR (antibiotic* ADJ3 resistan*) OR extended spectrum beta lactamase OR Pseudomonas aeruginosa OR Acinetobacter baumannii).ab,ti.) AND (((lower middle OR low middle OR low- and middle) ADJ6 income ADJ3 countr*) OR lmic OR lmics OR Armenia* OR Mongolia* OR Bhutan* OR Morocc* OR Bolivia* OR Nicaragua* OR (Cabo ADJ Verde*) OR Nigeria* OR Cameroon* OR Pakistan* OR Congo* OR (Papua New ADJ Guinea*) OR Cote d Ivoire OR Paraguay* OR Djibout* OR Philippin* OR Egypt* OR Samoa* OR Salvador* OR Sao Tome and Principe OR Georgia* OR Senegal* OR Ghan* OR Solomon Islands OR Guatemal* OR Guyana* OR (Sri ADJ Lank*) OR Hondur* OR Sudan* OR India OR Swaziland* OR Indonesia* OR Syria* OR Kiribati* OR Timor-Leste OR Kosov* OR Ukrain* OR Kyrgyz* OR Uzbek* OR Lao OR laos OR Vanuatu* OR Lesotho* OR Vietnam* OR Mauritania* OR (West Bank ADJ2 Gaza) OR Micronesia* OR Yemen* OR Moldova* OR Zambia*).kw,ab,ti. NOT (((exp child/ OR exp pediatrics/) NOT exp adult/) OR (pediatric* OR picu OR nicu OR picus OR nicus).ab,ti.)

Cochrane

((((('intensive care' OR 'critical care') NEXT/1 unit*) OR icu OR icus):ab,ti) AND ((infection* OR sepsis OR septic OR nosocomial* OR mrsa OR ((multidrug OR multi-drug OR resistan*) NEAR/3 (bacter*)) OR ((vancomycin OR methicillin OR carbapenem) NEAR/3 resistan*) OR vre OR mrsa OR esbl OR (antibiotic* NEAR/3 resistan*) OR 'extended spectrum beta lactamase' OR 'extended spectrum β lactamase' OR 'Pseudomonas aeruginosa' OR 'Acinetobacter baumannii'):ab,ti) AND ((('lower middle' OR 'low middle' OR 'low- and middle') NEAR/6 income NEAR/3 countr*) OR lmic OR lmics OR Armenia* OR Mongolia* OR Bhutan* OR Morocc* OR Bolivia* OR Nicaragua* OR (Cabo NEXT/1 Verde*) OR Nigeria* OR Cameroon* OR Pakistan* OR Congo* OR ('Papua New' NEXT/1 Guinea*) OR 'Cote d Ivoire' OR Paraguay* OR Djibout* OR Philippin* OR Egypt* OR Samoa* OR Salvador* OR 'Sao Tome and Principe' OR Georgia* OR Senegal* OR Ghan* OR 'Solomon Islands' OR Guatemal* OR Guyana* OR (Sri NEXT/1 Lank*) OR Hondur* OR Sudan* OR India OR Swaziland* OR Indonesia* OR Syria* OR Kiribati* OR 'Timor-Leste' OR Kosov* OR

Ukrain* OR Kyrgyz* OR Uzbek* OR Lao OR laos OR Vanuatu* OR Lesotho* OR Vietnam* OR Mauritania* OR ('West Bank' NEXT/2 Gaza) OR Micronesia* OR Yemen* OR Moldova* OR Zambia*):ab,ti NOT ((pediatric* OR picu OR nicu OR picus OR nicus):ab,ti)

Web-of-science

TS=(((((("intensive care" OR "critical care") NEAR/1 unit*) OR icu OR icus)) AND ((infection* OR sepsis OR septic OR nosocomial* OR mrsa OR ((multidrug OR multi-drug OR resistan*) NEAR/3 (bacter*)) OR ((vancomycin OR methicillin OR carbapenem) NEAR/3 resistan*) OR vre OR mrsa OR esbl OR (antibiotic* NEAR/3 resistan*) OR "extended spectrum beta lactamase" OR "extended spectrum β lactamase" OR "Pseudomonas aeruginosa" OR "Acinetobacter baumannii")) AND ((("lower middle" OR "low middle" OR "low- and middle") NEAR/6 income NEAR/3 countr*) OR lmic OR lmics OR Armenia* OR Mongolia* OR Bhutan* OR Morocc* OR Bolivia* OR Nicaragua* OR (Cabo NEAR/1 Verde*) OR Nigeria* OR Cameroon* OR Pakistan* OR Congo* OR ("Papua New" NEAR/1 Guinea*) OR "Cote d Ivoire" OR Paraguay* OR Djibout* OR Philippin* OR Egypt* OR Samoa* OR Salvador* OR "Sao Tome and Principe" OR Georgia* OR Senegal* OR Ghan* OR "Solomon Islands" OR Guatemal* OR Guyana* OR (Sri NEAR/1 Lank*) OR Hondur* OR Sudan* OR India OR Swaziland* OR Indonesia* OR Syria* OR Kiribati* OR "Timor-Leste" OR Kosov* OR Ukrain* OR Kyrgyz* OR Uzbek* OR Lao OR laos OR Vanuatu* OR Lesotho* OR Vietnam* OR Mauritania* OR ("West Bank" NEAR/2 Gaza) OR Micronesia* OR Yemen* OR Moldova* OR Zambia*) NOT ((pediatric* OR picu OR nicu OR picus OR nicus)))

Google scholar

"intensive|critical care"|icu|icus infection|infections|nosocomial|mrsa|vre|esbl|"lower middle-income country|countries"
|lmic|lmics|chine|egypt|indonesia|morocco|phillippines|algeria|bolivia|colombia|ecuador|guatemala|honduras|jamaica|nicaragua|thailand

Study eligibility

We followed the outlined stages of study selection guided by the aforementioned eligibility criteria (Figure 2). After retrieving by an experienced librarian, eligible papers (titles and abstracts) were exported to EndNote Library. The first author (YRS) screened all titles and abstracts and selected papers based on inclusion criteria. Another independent reviewer (HAV) performed a parallel review of titles and abstracts, and discrepancies between the two reviewers were resolved through consensus.

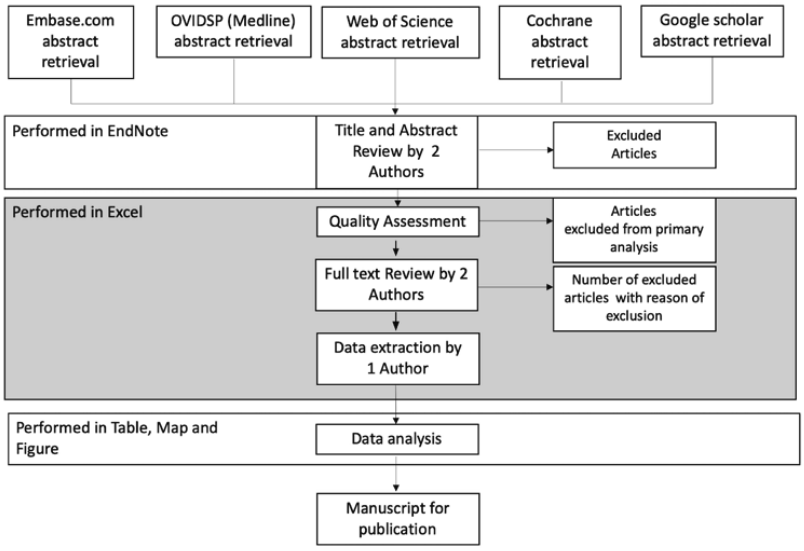


Figure 2. Overview of study methodology.

Subsequently, eligible papers published in journals ranked by their impact score as Q1 or Q2 in the Web of Science were selected for inclusion in the primary analysis. Full texts of the papers so selected were retrieved for full text review in a third round of screening for inclusion based on the criteria stated above, with reason for exclusion noted for each paper excluded on the basis of this full text review.

Papers excluded from the primary analysis based on the ranking of their journal of publication and those excluded during full text analysis were saved in separate files for potential analysis of specific questions arising during the remainder of the review process. Custom groups in EndNote were used to distinguish between various reasons for exclusion (Table 1), and articles were assigned to specific groups for certain sub-questions. The reviewers (YRS and HAV) worked in their own copies of this library. After reading all articles, each reference in the library was discussed in detail; therefore, no automatic comparison was used and any discrepancy was resolved.(5)

Data extraction

Data were extracted by first author (YRS) and inputted into a data extraction table (Excel) and independently checked by the senior author (HAV) to ensure quality. The extracted data comprised the characteristics of each study (first author name, year of publication, country, study

period and design), characteristics of hospital and adult ICUs, population characteristics, the type and characteristics of adult ICU-associated infection, laboratory diagnosis, the total and individual number of the species, Gram-negative and Gram-positive, isolated from patients, healthcare worker screening and environmental screening, their phenotypic and genotypic resistance characteristic, and the outcomes of patients (see Table 1).

Collecting and summarizing the findings

Thematic analysis was performed to identify the current etiology and management of nosocomial bacterial infections in adult ICUs in LMICs from the included studies.

RESULTS

Study selection

After duplicates were removed, a total of 1,961 citations were identified from searches of electronic databases (Figure 3). Based on the title and the abstract, 1,687 were excluded, with 274 eligible articles published in journals ranked by their impact score by the Web of Science. Of these 274 articles, 93 were published in Q1 or Q2 Journals and these 93 articles were subjected to a third round of eligibility check. Forty-two were excluded for specified reasons (see Figure 3 for reasons of exclusion) and the remaining 51 papers were included in this scoping review.

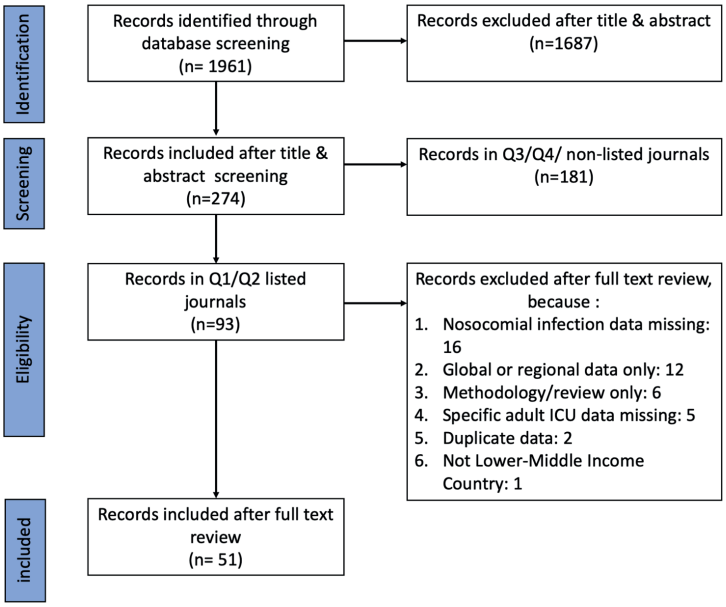


Figure 3. Flowchart for literature search

Geographical distribution and characteristics of included studies

All included studies were carried out in LMICs and published between 2005 and 2018. Fifty-one qualified studies were conducted in South Asia (India: 22 studies (6-27), Pakistan: 2 (28, 29)), Middle East & North Africa (Egypt: 9 (30-38), Morocco:2 (39, 40)), East Asia & Pacific (Vietnam: 6 (41-46), Indonesia: 2 (47, 48), Philippines: 2 (49, 50), Mongolia: 1 (51)), Sub-Saharan Africa (Nigeria: 2 (52, 53), Ghana: 1 (54)), and Europe & Central Asia (Kosovo: 2 (55, 56)) (Figure 4). Thus, the majority of LMICs did not have information on ICU-associated infections published in Q1 or Q2 Journals in this time frame. Most publications described surveillance and observational studies, only ten publications reported on intervention studies, either randomized or quasi-experimental in design. Multicenter studies were described in 28 publications.

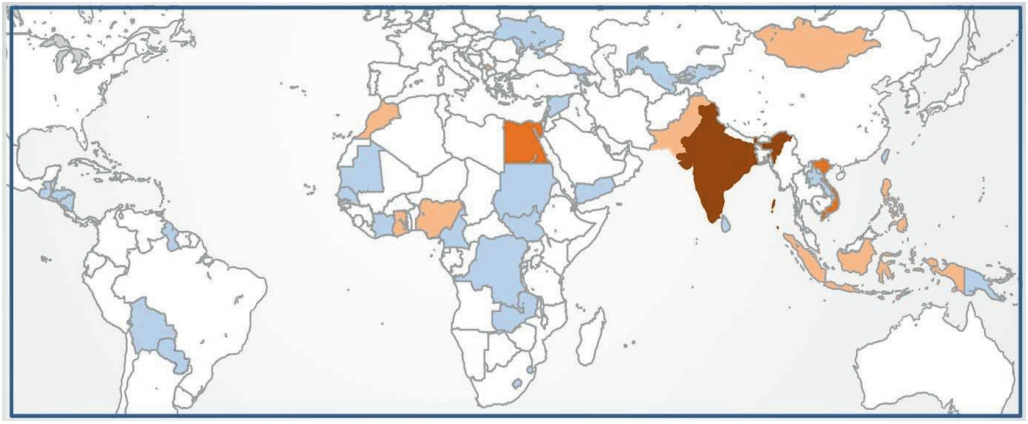


Figure 4. LMIC highlighted by number of studies reporting on Intensive Care Unit-associated infections in 2005-2018.

Characteristic of ICUs were not same, because some ICUs were highly specialized including Burn ICUs or Liver and post-transplant ICUs; however, most were mixed medical-surgical units with an open ward design. The number of beds per ICU ranged between 4-90. The majority of patients were male (38-79%). Eleven studies presented the median of age of patients admitted, it ranged from 25-61 years, with a mean of the medians of 53 years. Twenty-three studies presented the mean age of patients admitted, it ranged from 32-71 years, with a mean of the means of 50 years. In the contemporary EPIC II study, the mean age of patients admitted to ICUs was 60.7 years.(57)

ICU infection rate and outcomes

The overall frequency of ICU infections was presented using three types of calculations, as an attack rate in 11 reports, as point prevalences in eight and as incidence rates in seven, with some reports using multiple measures (Table 2). The overall attack rate was 9.1 infections/100 admissions, and varied between 4.4-129.3/100 admissions. (7, 9, 22, 30, 39, 43, 44, 52, 53, 55, 56) We identified point prevalence data in 8 studies, overall it was 22.4 infected patients/100 admitted patients, and varied between 8.5-50. (7, 22, 36, 37, 47, 52, 53, 56). The overall incidence rate was 9.1 infections/1,000 patient-days, based on data from 7 studies, it varied between 2.4 and 79 infections/1,000 patient-days in the ICU (7, 9, 22, 35, 37, 38, 53). Expressed as device specific incidences, ventilator-associated pneumonia (VAP) occurred at a rate of 11.3 episodes/1,000 days on ventilation, central line-associated bloodstream infection (CLABSI) at 4.1 episodes/1,000 days with central line and catheter-associated urinary tract infection (CAUTI) at a rate of 3.0 episodes/1,000 days with urinary catheter (Table 2).

The median lengths of stay were presented in 15 studies (7, 22, 24-26, 32, 41, 42, 44, 46, 48, 52-54, 56), it ranged between 5-17 days. We calculated an overall median of the medians length of stay of 11 days, and an overall mean of the medians length of stay of 10 days. The overall in-ICU mortality rate extracted from 18 studies was 33.6% (1,753/5,241) patients. If we looked at individual studies, we found a wide range in recorded mortality rates varying between 14% and 70%. (7, 11, 14, 19, 24-27, 31, 35, 40, 46, 48, 53, 54).

Etiology of infection acquired in ICU

Information on pathogens causing all ICU-associated infections was available from 11 studies (7, 10, 22, 26, 30, 37, 41, 44, 46, 52, 55), six studies included microbiological data specifically related to VAP (7, 8, 20, 22, 52, 53), seven had data related to CAUTI (7, 8, 22, 31, 50, 52, 53), and six to CLABSI (7, 8, 15, 22, 52, 53).

Gram-negative bacilli constituted the most prevalent group of nosocomial pathogens in these ICUs. The most common single pathogen causing ICU-acquired infection in LMICs were *A. baumannii* (24%)*, P. aeruginosa* (16%)*,* and *K. pneumoniae* (15%)*,* these caused the majority of infections. This distribution of pathogens is significantly different from the distribution of pathogens causing ICU-acquired infection in West European countries in the same period, where these same three species caused <25% of all infections.(57) In the European setting, Gram-positive pathogens were more prominent and the group of other nosocomial agents of ICU infection was larger (Figure 5).

Table 2. Infection rates in Intensive Care Units in Lower-Middle Income Countries, 2005-2018.

biostatistical measure	patients admitted to ICU	patients infected during ICU stay	ICU-acquired infections	total days stayed in ICU	observed frequency	references
			number			
attack rate	22,403		2,032		9.1/100 admissions	(7, 9, 22, 30, 39, 43, 44, 52, 53, 55, 56)
point prevalence	2,129	476			22.4/100 admitted	(7, 22, 36-38, 47, 52, 56)
incidence rate			3,614	397,307	9.1/1,000 ICU days	(7, 9, 22, 35, 37, 38, 53)
VAP rate			1,404 VAP	124,393 days on ventilator	11.3/1.000 days on ventilator	(8-10, 16, 19, 20, 22, 26, 32, 33, 35, 38, 42, 51)
CLABSI rate			1,053 CLABSI	255,828 days with central line	4.1/1.000 days with central line	(8-10, 15, 19, 22, 26, 27, 32, 33, 35, 38, 49, 51)
CAUTI rate			916 CAUTI	300,679 days with catheter	3.0/1,000 days with catheter	(8-10, 19, 22, 26, 31-33, 35, 38, 42, 49-51)

A. baumannii was the most frequent pathogen identified for ventilator-associated pneumonia causing 42% of VAP, followed by *P. aeruginosa* which caused 25% of the VAP. Thus, these two species were involved in two thirds of all episodes of VAP in LMIC (Figure 6). In contrast, *K. pneumoniae* was the dominant species in CLABSI, causing 24% of the episodes, as much as the combined impact of *A. baumannii* and *P. aeruginosa.* Together, the ESKAPE species were involved in two thirds of all CLABSI episodes. ESKAPE species also caused 51% of CAUTI in this setting, with *Escherichia coli* as the most prevalent representative species. However, a sizable minority of CAUTI were caused by other species of uro-pathogenic microorganisms including many episodes that were caused by *Candida* species (data not shown).

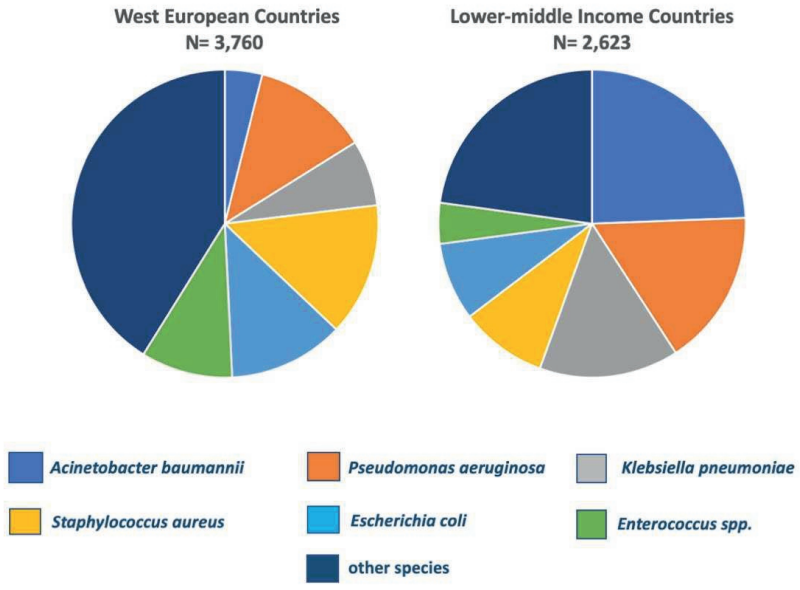


Figure 5. Distribution of ESKAPE pathogens causing ICU acquired infection in LMICs and in West European countries

Legend: ESKAPE pathogens include *Enterococcus spp., Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Escherichia coli.* Data from West European countries were extracted from reference (57).

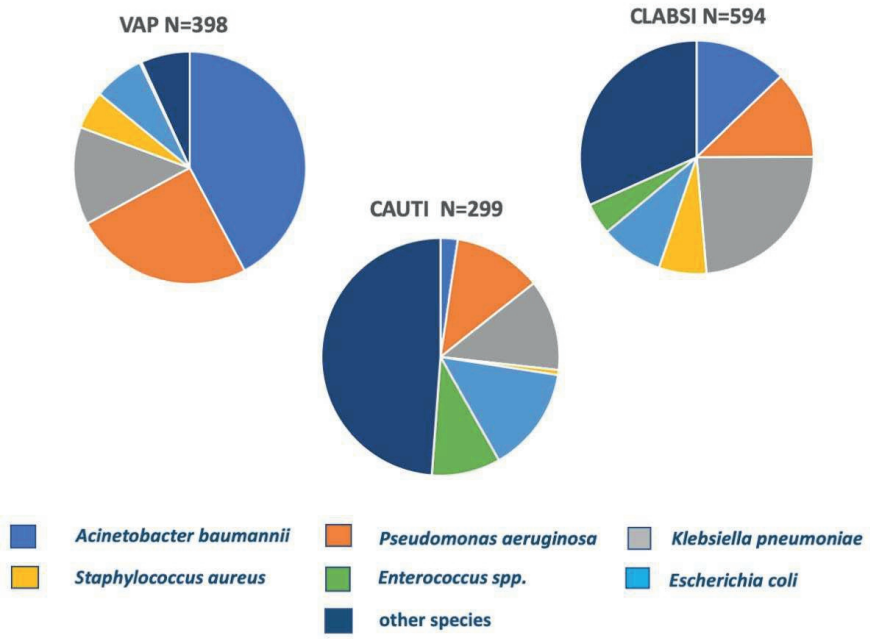


Figure 6. Distribution of ESKAPE pathogens causing Ventilator Associated Pneumonia (VAP), Catheter Associated Urinary Tract Infection (CAUTI) and Central Line Associated Bloodstream Infection (CLABSI) in ICUs in Lower-Middle Income Countries, 2005-2018.

Legend: ESKAPE pathogens include *Enterococcus spp., Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Escherichia coli.*

Phenotypic susceptibility pattern

Phenotypic resistance profiles of ESKAPE isolates to various antibiotics was determined in 15 studies. However, these studies were reported from only six Lower-Middle Income Countries, and were sometimes lacking data on certain combinations of ESKAPE species and classes of antimicrobial agents. Almost all isolates from LMIC were resistant to multiple classes of antibiotics, a condition that closely resembles the resistance levels observed in some Mediterranean countries of West Europe (Table 3). Compared to LMIC isolates the same species isolated from invasive infections in Nordic countries of West Europe displayed much lower levels of antibiotic resistances (Table 3). Vancomycin resistance among *Enterococcus* species was >50% in Vietnam (44) and *MRSA* (Methicillin resistant *Staphylococcus aureus)* was identified in >50% of all *S. aureus* isolates in most LMIC (22, 24, 29, 44, 53). Multidrug resistant *K. pneumoniae, A. baumanii,* and *P. aeruginosa* was found among >50% of the isolates in India, Pakistan, Egypt, Vietnam and Nigeria (7, 14, 21, 22, 29, 34, 36, 41, 46).

Genotypic resistance pattern

Only a very few studies presented genetic information regarding the antibiotic resistances observed. Amissah *et al.* from Ghana reported that 28% isolates of *S. aureus* tested positive for the *mec*A gene (54). Carbapenemase genes ($bla_{\text{OXA-23}}$, $bla_{\text{OXA-51}}$, $bla_{\text{OXA-66}}$, $bla_{\text{OXA-68}}$) in *A. baumannii* were characterized in 4 studies, in Indonesia (48), Egypt (34, 36) and Morocco (40).

Environment screening culture

Environmental screening cultures were performed and reported in four separate studies only. Taneja, N. et al. in 2005 in India (6) collected 178 environmental samples from various sources and fluids in their main and transplant ICUs and found 51(28.7%) to be contaminated with potential pathogens, of which 31 (20.9%) were contaminated with Gram-positive bacteria, 26 (14.6%) with Gram-negative bacilli and 11 (6.2%) with fungi. Gupta, M. et al. (21) more recently reported the presence of *A. baumannii* in 17/26 (65%) samples of humidifier water, and in 3/6 (50%) heat and moisture exchangers cultured in their ICU in a tertiary care center in South India. These environmental isolates showed the same multidrug resistance pattern as contemporary isolates from patients admitted to the ICU.

In Morocco Uwingabiye, J. et al (40) identified 36 environmental *A. baumannii* isolates and compared them with 47 clinical isolates of the same species. They showed genetic similarity between the clinical and environmental isolates since 80/83 (96.4%) of all isolates belonged to the same 7 PFGE pulsotypes. Saharman, Y.R. et al (48) likewise found six isolates of carbapenem-non-susceptible *A. baumannii-calcoaceticus complex* in the environment of two ICUs in a tertiary care center Indonesia, four of these isolates belonged to same dominant MLST clone infecting their patients.

Health care worker screening

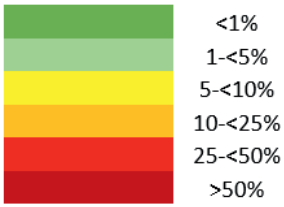
Health care workers (HCW) may be another source of nosocomial pathogens, thus HCW screening may be an important measure to detect and eradicate such sources of antimicrobial resistance. However, only two studies addressing HCW carriage of resistant pathogens were available from LMIC in this time frame, one from Indonesia (48) and one from Ghana (54). Saharman, Y.R et al (48) identified one HCW in their ICUs that carried carbapenem-non-susceptible *A. baumannii-calcoaceticus complex,* and Amissah et al (54) found colonization with *S. aureus* isolates were obtained from 13/29 (45%) of their HCWs, but only one of which carried MRSA.

Table 3. Phenotypic susceptibility patterns of ESKAPE species causing ICU infection in Lower-Middle Income Countries (LMIC) compared to susceptibilities of the same species causing invasive infections in indicated European Union (EU) countries, 2005-2018.

Species	Antibiotic	EU-Sweden	EU-Netherlands	EU-France	EU-Italy	EU-Greece	LIMC-India	LIMC-Pakistan	LMIC-Egypt	LMIC-Vietnam	LMIC-Nigeria	LMIC-Morocco
Enterococcus spp.	Vancomycin											
S. aureus	Methicillin											
K. pneumoniae	Aminoglycoside											
K. pneumoniae	Fluoroquinolone											
K. pneumoniae	3rd gen. cephalosporins											
K. pneumoniae	Carbapenems											
A. baumannii	Aminoglycosides											
A. baumannii	Fluoroquinolones											
A. baumannii	Carbapenems											
P. aeruginosa	Aminoglycosides											
P. aeruginosa	Ceftazidime											
P. aeruginosa	Piperacillin/tazo											
P. aeruginosa	Fluoroquinolones											
P. aeruginosa	Carbapenems											
E. coli	Aminoglycosides											
E. coli	Fluoroquinolones											
E. coli	3rd gen. cephalosporins											
E. coli	Carbapenems											

Note:

Data from indicated European Union countries were derived from reference (57). Colors indicate increasing levels of resistance as specified in the legend, and blank boxes indicate that no data was available for the particular combination of species and antimicrobial agent.



Intervention study

We identified 10 publications that described interventions aimed to reduce ICU acquired infections and antimicrobial resistances; all but one applied a quasi-experimental design to measure the effects of their intervention (Table 4) (12, 15-18, 27, 28, 42, 43, 50). Multimodal strategies (those with ≥3 components implemented in an integrated manner to achieve improved outcomes and change behavior as defined by WHO guidelines) were used in most studies.(58) Outcomes were either processes, especially hand hygiene practice, in five studies or actual rates of ICU acquired infections in seven studies (two studies had both types of outcomes, Table 4). Thu et al. (2015) in Vietnam performed a cost-effectiveness study analyzing the impact of a HH improvement program in ICUs. The study used the steps recommended by the WHO, including upgrading HH facilities, training, surveillance, and feedback. The study showed that HH compliance increased from 25.7% to 57.5% and the incidence of HAI decreased from 31.7% to 20.3% (P<.001) after the intervention; similar results were shown in several reports from India (12, 17, 18).

Successful interventions have also targeted CLABSI, VAP, and CAUTI. The implementation of a multidisciplinary approach for prevention of VAP in ICUs in Pakistan (28) showed reduction from 18% to 13% in the VAP rate, and in India (16) from 17.4 to 10/1,000 ventilation days. In 16 ICUs in India a similar intervention strategy for CLABSI also showed a reduction in CLABSI incidence rates from 6.4 to 3.9/ 1000 central line days.(15) Finally, Navoa-Ng, J. A. et.al in the Philippines targeted CAUTI and reported a reduction of CAUTI from 11.0 to 2.66/1,000 urine-catheter days as a consequence of applying an infection prevention bundle together with education, monitoring and feedback.(50)

DISCUSSION

In this systematic scoping review, we have shown that endemic nosocomial infections represent a major burden and safety issue for patients admitted to intensive care in the lower-middle income countries. Unfortunately, there were relatively few studies published in a highly qualified scientific Journals (Q1/Q2 by Web of Science rank) on this topic. From 50 LMICs countries, we only identified 51 qualified published studies published from 11 LMICs over a thirteen-year time frame. There is, thus, a need to obtain a more comprehensive view on the problems of LMICs to control ICU acquired infection and resistance to antimicrobial agents in such settings.

The ICU cannot be rendered sterile but every effort should be made to reduce the numbers of ICU-acquired healthcare-associated infection (HAI) and the risk of spread of resistant nosocomial pathogens. Strategies to minimize infection have been incorporated into various guidelines on ICU design that are available in the UK, the USA and Europe.(59, 60) An ICU should

accommodate at least 6 beds with 8–12 beds considered as the optimum. Hospitals with several smaller units should be encouraged to rearrange these units into a single larger department to improve efficiency. A larger ICU may provide opportunities to create separate, specialized functional subunits with 6–8 beds, sharing the same geographical, administrative, and other facilities. (59, 60). However, of those described most ICUs in LMICs still had open ward designs with one large room, with beds separated by curtains only, they did not have separate cubicles or separate isolation rooms. The numbers of beds ranged between 4-90 beds. These open ICU designed are not optimal, they compare unfavorably with the current trend to construct ICUs as a series of separate rooms to better protect patients against ICU acquired infections (59). Also, not all ICUs in LMIC had dedicated and qualified intensivists; however, most of them did have multidisciplinary teams in charge of the patients (data not presented).

The ICU-acquired infection rates were quite high in LMIC, with an average point prevalence rate of 22.4 infected patients per 100 presences to the ICU. This rate is comparable, albeit somewhat higher, to the average point prevalence rate of 19.5% recorded in ICU across West European countries in the same time frame (2011-2012) (61). The devices associated infection indices were also comparable to those recorded in West European ICUs at that time, 9.5 VAP/ 1,000 intubation days, 3.3 CLABSI/ 1,000 days with central line and 4.5 CAUTI/1,000 days with urinary catheter.(62) Thus, the overall impression is that ICU acquired infections in LMIC are quite similar in their nature, but that rates are somewhat higher (approximately 15%) in LMIC ICUs compared to ICUs in West European countries.

ICU length of stay and ICU mortality are important outcomes of intensive care. In studies retrieved by our search, the overall length of stay was 10 - 11 days and the overall ICU mortality rate was 33,6% (varying from 14 -7 0% across the studies). In the same time frame in European countries, based on ICU surveillance from 2008-2012, the median (IQR) length of stay was 10 (8-12) days, which was highly comparable to the length of stay in ICUs in LMIC.(62) However, mortality rates differed significantly. On average, 15.3% of EU patients staying more than two days died in the ICU, ranging from 8.7% in Luxembourg to 18.1% in France.(62) The Extended Prevalence of Infection in Intensive Care (EPIC II) study (2007) involving 1,265 ICUs and 75 countries was found an overall ICU mortality rate of 18.2% (2,370/13,011 patients). Infected patients had higher ICU mortality rates (25.3%) and longer ICU lengths of stay (16 days[IQR, 7-34].(57, 62) Thus, the overall ICU mortality rate of 33.6% retrieved in this scoping review was much higher in LMIC, indicating that, compared to high income countries, patients in ICU's in LMIC die at a higher rate and that death comes relatively early during their ICU stay. The fact that in LMIC the mean age of ICU patients was much lower than in high income countries (50 years versus 60 years) further underscores the major discrepancy in ICU survival between these two groups of countries

Table 4. Intervention Studies Performed in Lower-Middle Income countries 2005-2018.

Study	study period	published year	country	hospitals	ICUs	objective	study design	intervention	subjects or observations	outcomes	comments
Khan,MS (28)	7/2006-11/2007	2009	Pakistan	1	1	reduce VAP	quasi-experimental before/after study	6 h training only	582 MV patients	VAP rate/ 100 MV patients from 18% to 13% (p=0.11).	all patients were surgical and ventilated; MDR *A. baumannii, P. aeruginosa* and *K. pneumoniae* most prevalent
Mathur P (12)	7/2010-9/2010	2006	India	1	1	increase HH compliance	quasi-experimental before/after study	questionnaires, education & training, monitoring	1,489 HH opportunities	compliance from 8.4% to 63.1% (p<0.0001). Housekeeping staff did not increase their HH-compliance	small scale, short term study; not clear whether housekeeping was trained or not.
Jaggi N (15)	9/2004-2/2012	2013	India	11	16	prevent CLABSI by multidimensional approach	quasi-experimental before/after study	infection prevention bundle, education, monitoring & feedback	35,650 patients yielding 90,370 CL days	CLABSI/1,000 CL days from 6.4 to 3.9 for a RR of 0.61(0.46-0.81) p=0.0007. Less *S. aureus* after intervention but more *P. aeruginosa. K. pneumoniae* most prevalent pathogen throughout study.	mean age was 1.3 year higher in intervention period

Study	study period	published year	country	hospitals	ICUs	objective	study design	intervention	subjects or observations	outcomes	comments
Mehta Y (16)	7/2004-10/2011	2013	India	14	21	prevent VAP by multidimensional approach	quasi-experimental before/after study	infection prevention bundle, education, monitoring & feedback	46,945 patients yielding 65,574 MV days	VAP/100 MV days from 17.4 to 10.8 for a RR of 0.62 (0.50-0.78), p=0.0001.	patients had little lower ASIS scores in intervention period
Navao-Ng JA (50)	12/2005-12/2010	2013	Philippines	2	4	preventing CAUTI by multidimensional approach	quasi-experimental before/after study	infection prevention bundle, education, monitoring & feedback	3,183 patients yielding 8,720 urinary catheter days; observed HH opportunities 4,191	CAUTIs/1,000 UC days from 11.0 to 2.66 for a RR 0.24 (0.22-0.53); HH compliance from 57.2 to 78.2% (RR 1.37[1.21-1.54])	few HH opportunities in baseline period
Schultsz C (42)	5/2004-4/2006	2013	Vietnam	1	1	prevent exogenous acquisition of MDRO	quasi-experimental before/after study	hand hygiene reinforcement, revising infection prevention procedures, monitoring & feedback, adjust antibiotic policy	357 patients	VAP/1,000 MV days from 56 to 40, UTI/1,000 UC days from 12.8 to 15.0 (both not significant). Less cephalosporins, penicillin and carbapenem and more fluoroquinolones, metronidazole and broad-spectrum penicillin used; only MRSA acquisition delayed, not seen for other MDRO.	patients had more severe tetanus + more MV days + longer LOS in year 2; HH compliance only measured in year 2.

Study	study period	published year	country	hospitals	ICUs	objective	study design	intervention	subjects or observations	outcomes	comments
Biswal M (17)	11/2010-5/2013	2014	India	1	7	improving hand hygiene	quasi-experimental before/after study	repeated education & training, posters, adequate supplies of alcohol & soap	3,212 HH opportunities	HH-compliance up from 16.5% to 28.2% and 35.1 % after 1st and 2nd training week respectively. Significant in all ICUs.	low numbers of opportunities per ICU
Chakravarthy M (18)	8/2004-7/2011	2015	India	3	3	improving hand hygiene by multidimensional approach	quasi-experimental before/after study	allocation supplies, education & training, reminders, monitoring & feedback	3,612 HH opportunities	HH compliance up from 36.9% to 74.8% for a RR 2.0 (1.7-2.4), p=0.0001; but not in surgical ICU? poor among ancillary staff; HH improvement maintained over 3 years.	only few observations in surgical ICU
Thu LTA (43)	6/2009-4/2011	2015	Vietnam	1	17	reducing HAI by hand hygiene promotion	quasi-experimental before/after	questionnaires, education & training (including patients & visitors), posters & flyers, new sinks, hand alcohol made available	984 patients and 6,046 HH observations	HAI/100 pts: from 31.7% to 20.3% (p=0.005), all HAI types; hand hygiene compliance from 25.7% to 57.5% (p<0.001)	

Study	study period	published year	country	hospitals	ICUs	objective	study design	intervention	subjects or observations	outcomes	comments
Rosenthal VD (27)	4/2012-8/2014	2015	India	2	5	CLABSI reduction	RCT, block-randomization	introduced new IV flush device	1,096 patients yielding 7.680 CL days	CLABSI/1,000d: 2.21 vs 6.40; RR 0.35(0.16-0.76); cost effective, Qualys-increasing; shift in microbe species	

Abbreviations: VAP (ventilator-associated pneumonia), MV (mechanical ventilation), RR (risk ratio), MDR (multidrug-resistant), CAUTI (catheter-associated urinary tract infection), UC (urinary catheter), HH (hand hygiene), MDRO (multidrug-resistant organism), CLABSI (central line-associated blood stream infection), CL (central line), HAI (hospital-acquired infection), RCT (randomized controlled trial), IV (intravenous).

Gram negative bacteria were responsible for more than 50% of the total number of ICU acquired infections recorded in LMIC. This distribution contrasts with findings from studies done in Western Europe at that time where the prevalent cause of health care-associated infections had switched over to Gram-positive microorganisms (72.7%) (EPIC II study).(57) The microorganisms most frequently isolated from ICU infections in a later study (61) were in decreasing order, *Escherichia coli* (15.9%) ,*Staphylococcus aureus* (12.3%), *Enterococcus spp*. (9.6%), *Pseudomonas aeruginosa* (8.9%) *Klebsiella spp*.(8.7%), Coagulase-negative *staphylococci* (7.5%), *Candida spp*. (6.1%), *Clostridium difficile* (5.4%), *Enterobacter spp.* (4.2%), *Proteus spp.* (3.8%) and *Acinetobacter spp.* (3.6%).(61) Especially the proportion of infections caused by *Acinetobacter spp.* in ICUs in LMIC countries was more than six times higher compared West European countries (24% versus 3.6%).(57)

The ESKAPE group of pathogens will be of increasing relevance to antimicrobial chemotherapy in the coming years. Our findings revealed a high rate of multi-drug resistant (MDR) Gram-negative bacilli causing ICU infections in LMIC. The high percentages of resistance to third generation cephalosporin and of multidrug resistance in Gram-negative bacteria remain worrisome. Comparably high rates of MDR among Gram-negative bacilli isolated from patients with invasive infections in Italy, Greece and some in France (EARS-Net by 30 EU/EEA countries in 2014).(63) Discordantly, much lower MDR rates among Gram-negative bacilli were observed from invasive infections in Sweden and the Netherlands.(63) The high percentages of resistance to carbapenems of *P. aeruginosa, A. baumannii* and *K. pneumoniae* isolates found in this scoping review reflect the challenges of treatment of ICU patients in LMIC.

The implementation of a multidisciplinary approach for prevention of HAIs in ICUs from LMICs showed that reductions in the HAI rate are possible in LMIC. Some studies reported effective interventions including contact precautions, active surveillance cultures, monitoring, audit and feedback of preventive measures, patient isolation or cohorting, hand hygiene improvement programs, and environmental cleaning. This is also highlighted by the recent evidence-based WHO Guidelines on core components of IPC programs, which strongly recommend multimodal strategies to translate IPC measures into clinical practice.(58)

One of the most comprehensive guidelines is the 2013 European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Guidelines for the management of infection control measures to reduce transmission of multidrug-resistant (MDR) Gram-negative bacteria. In endemic settings, hand hygiene and contact precautions were the only two interventions that were strongly recommended for all three pathogens (MDR-*K. pneumoniae*, MDR-*P. aeruginosa*, and MDR-*A. baumannii*) in addition to isolation for MDR-*K. pneumoniae* and isolation, alert codes, education, and environmental cleaning for MDR *A. baumannii*. In epidemic settings, hand hygiene, contact precautions, active screening, isolation and, last but not least, environmental cleaning are

strongly recommended for all three pathogens in addition to alert codes and cohorting for MDR-*K. pneumoniae*.(64) Interestingly, implementation of hand hygiene best practices and environmental cleaning was reported in only few studies in LMICs so far. Effective hand hygiene compliance is widely recognized and strongly recommended by WHO to reduce transmission of pathogenic microorganisms in healthcare. Likewise, the important role of the innate environment of the ICU providing sources and routes of transmission of multidrug resistant microorganisms is gaining recognition worldwide. This scoping review revealed that implementation of these guidelines is essentially possible in LMIC, and are sorely needed to reduce the high burden of disease caused by ICU acquired infections in these settings. Much room for further high quality observational and interventional research remains that should include more countries among the LMIC, and target novel interventions that are cost-effective in this particular setting.

A limitation of this review is posed by the relatively low number of qualified studies that were performed in only a minority of the 50 countries belonging to group of LMIC. Thus, this review cannot be taken to reflect the full scope of ICU acquired infections in all LMIC, but from our perspective this currently represents the best available view on infections acquired in ICUs in Lower-Middle Income Countries.

CONCLUSIONS

Our systematic scoping review describes the current evidence of ICUs acquired infections in LMICs countries. Many gaps in knowledge remain since most LMIC have not produced high quality reports. However, from the reported evidence it is clear that the rate of ICU acquired infections is likely to be somewhat higher in LMIC compared to High Income countries and that the ICU mortality rate is much higher. Multidrug resistant Gram-negative bacilli, especially *Acinetobacter spp.* and *Pseudomonas spp.* from the environment seem to play a much more dominant role in LMIC than in High Income countries. However, interventions to improve this situation have been shown to be feasible and effective, even cost-effective.

DECLARATIONS

Ethics and Regulatory Considerations

The scoping review protocol was developed as recently recommended by PRISMA extension for scoping reviews and registered in the Open Science Framework, an international prospective register of systematic scoping reviews on 13th December 2019 (https://osf.io/c8vjk).

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

YRS is an awardee of the DIKTI-NESO Scholarship by The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, and Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands.

All authors report no conflict of interest relevant to this article.

Funding

This work was supported by 'The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia' and 'Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands'.

Authors'contributions

YRS, HAV, and JAS conceived the study and participated in design of the study.

YRS, and HAV performed data analysis and interpreted the data.

YRS, AK, HAV, and JAS drafted the article.

All authors participated in critically revising the draft.

All authors read and approved the final manuscript.

Acknowledgements

We are thankful to The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, Dean of Faculty of Medicine Universitas Indonesia, Board of Directors of Dr. Cipto Mangunkusumo National General Hospital Jakarta Indonesia, Department of Medical Microbiology and Infectious Diseases,

We are special thanksful to Dr. Wichor M.Bramer, PhD (Biomedical information specialist (literature research, esp. Systematic Reviews)) from Erasmus MC Medical Library, for his assistance with the development of the systematic review search strategy and retrieving the eligible studies.

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CHAPTER 3

3

Endemic Carbapenem-Nonsusceptible *Acinetobacter baumannii-calcoaceticus* Complex in Intensive Care Units of the National Referral Hospital in Jakarta, Indonesia

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ABSTRACT

Background:

Carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex have emerged worldwide, but the epidemiology in Indonesian hospitals has not been studied.

Methods:

A prospective observational study was performed on the intensive care units (ICUs) of the national referral hospital in Jakarta-Indonesia, in 2013 and 2014. All consecutive adult patients admitted and hospitalized for >48 hours in ICUs were included. Basic and clinical data at admission were recorded. carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex from clinical cultures and standardized screening were included. Environmental niches and healthcare workers (HCWs) were also screened. PCR was used to detect carbapenemase genes, and Raman spectroscopy as well as multilocus sequence typing (MLST) for typing.

Results:

Of 412 included patients, 69 (16.7%) carried carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex on admission, and 89 (25.9%) became positive during ICU stay. The acquisition rate was 43 per 1,000 patient-days at risk. Six isolates were cultured from environment and one from a HCW. Acquisition of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex was associated with longer ICU stay (median interquartile range [IQR]: 11 days [5-18], adjusted hazard ratio [aHR]: 2.56 [99% confidence interval (CI):1.76-3.70]), but not with mortality rate (adjusted odds ratio: 1.59 [99%CI: 0.74-3.40] at the chosen level of significance). The $bla_{\text{OXA-23}}$-like gene was detected in 292/318 (91.8%) isolates, including isolates from the environment and HCW. Typing revealed five major clusters. Sequence types (ST)195, ST208, ST218, ST642 as well as new STs were found. The dominant clone consisted of isolates from patients and environment throughout the study period.

Conclusions:

Carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex are endemic in this setting. Prevention requires source control and limiting transmission of strains.

Trial registration:

The study was retrospectively registered at www.trialregister.nl (No:5541). Candidate number: 23527, NTR number: NTR5541, Date registered NTR: 22nd December 2015

Keywords:

Acinetobacter baumannii-calcoaceticus complex, Intensive Care Unit, Carbapenems, Antimicrobial resistance, Carbapenemase, Indonesia.

INTRODUCTION

Multidrug-resistant *Acinetobacter baumannii-calcoaceticus* complex has emerged as one of the most problematic pathogens in hospitals. Their natural habitat is in the environment, including niches in the hospital from which they can spread to patients. [1] Risk factors for colonization and infection with multidrug-resistant *A. baumannii-calcoaceticus* complex include length of hospital stay, admission to an intensive care unit (ICU), mechanical ventilation, antimicrobial exposure, and several other factors. [2] Carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex is considered a significant health problem because of the limited options remaining for antibiotic treatment. [3]

In 2013, the Centers for Disease Control and Prevention of the United States reported an estimated 12,000 healthcare-associated *Acinetobacter* infections. Nearly 7,000 of these were caused by multidrug-resistant isolates. [4] In 2008, Lagamayo *et al*. reported that between 2-77% of all clinical isolates of *Acinetobacter* spp. in Asian countries were resistant to imipenem, and that multidrug-resistant *Acinetobacter* spp. were highly prevalent, particularly in Thailand and India, but not in the Philippines [5].

To date, there have been no data on the epidemiology of carbapenem-resistant or -nonsusceptible *A. baumannii-calcoaceticus* complex from Indonesia, the fourth most populous country in the world. This study was designed to delineate the clinical and molecular epidemiology of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex in two ICUs of the national referral hospital in Jakarta, Indonesia.

PATIENTS AND METHODS

Study Design

A prospective observational study was performed at the Dr. Cipto Mangunkusumo General Hospital a 1,000-bed teaching hospital in Jakarta, Indonesia, from April-October 2013 and from April-August 2014. We conducted this study in two ICUs: the 12-bedded adult ICU and the 8-bedded Emergency Room (ER)-ICU with an average of 1,010 and 415 admissions per year, respectively. The adult ICU is an open ward with mechanical ventilation facilities, admitting patients with mixed medical and surgical indications, and one designated nurse per patient during first shifts (7am-3pm) and a 1:1.5 nurse/patient ratio during other shifts. The ER-ICU has the same design, and the nurse-to-patient ratio in the morning shifts is 1:1 and during the other shifts 1:2. The populations served by these two ICUs were identical, and there was also no difference in the service provided.

The study was performed in the framework of a larger study that focused on carbapenem-nonsusceptible *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *A. baumannii-calcoaceticus* complex. All adult patients (≥18 years old) admitted to one of the two ICUs and hospitalized for

more than 48 hours were eligible for enrollment in this study. The first screening cultures were taken on the day of admission, and if a patient was discharged before 48 hour, he or she was excluded. Informed consent was obtained from the patient or their relatives as applicable. Demographic and clinical characteristics such as age, gender, medical or surgical indication, underlying diseases, hospitalization history, and previous use of antibiotics were recorded on admission.

Systemic inflammatory response syndrome (SIRS) criteria on admission were used as a screening tool to assess (severity of) septic illness. The SIRS criteria were calculated and included in the study, as this was practice at the time of the study.[6]

The quick Sequential Organ Failure Assessment (qSOFA) score is a new bedside prompt that may identify patients with suspected infection and helps to determine sepsis in all healthcare environments. The qSOFA score assigns one point for each of the following conditions: systolic blood pressure ≤100 mmHg, respiratory rate ≥22 breaths per minute, and altered mentation (Glasgow coma scale <15). A qSOFA score ≥2 at the onset of infection is associated with a greater risk of death and prolonged ICU stay. [6]

The primary outcome measure was acquisition of a carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex. Acquisition is defined as a screening culture or clinical culture with first detection of *A. baumannii-calcoaceticus* complex, with reduced susceptibility to a carbapenem, that was not present within the first 48 hours of admission. Secondary outcome measures were length of stay in the ICU, and mortality during ICU stay.

Environmental samples (Supplementary Table 1), were taken twice (in October 2013 and December 2014), simultaneously in both ICUs. Screening of healthcare workers (HCWs) was performed once. HCWs were defined as all personnel including doctors, nurses and other people (cleaning staff, administration staff, porters, nutritionist) working in one of the two ICUs during the study period.

Screening Method

From patients enrolled, screening cultures were obtained from throat and rectum or stools by experienced ICU nurses who had been trained for the task of taking the samples, on the day of admission, at the time of discharge from the ICU, and weekly if the patient was admitted for seven days or more. Sampling was performed using sterile cotton-tipped swabs, and swabs were transported to the laboratory in Amies transport medium (Oxoid, Basingstoke, UK). The swabs were transported in clean, closed boxes at ambient temperature to the laboratory on the same day. All swabs were processed in the laboratory within 24 hours.

Clinical samples were collected on indication from patients under aseptic precautions from the lower respiratory tract, blood, urine, tissue, or wound. Environmental samples were

taken from various sites, including wash basins, bed rails, bedside cabinet tables, ventilators, and monitor screens (Supplementary Table 1), with sterile cotton-tipped swabs and placed in Amies transport medium. All HCWs working in one of the ICUs were sampled once over the course of one month (September 2013) with sterile cotton-tipped swabs, which were transported to the laboratory in Amies transport medium.

Microbiological Methods

Isolation and Identification

In the Clinical Microbiology Laboratory of Faculty of Medicine Universitas Indonesia, Jakarta, each swab was placed in 5 ml trypticase soy broth (TSB) supplemented with cefotaxime 2 mg/L plus vancomycin 50 mg/L and incubated overnight. The next day, a loop of broth (10 µl) was subsequently subcultured onto MacConkey agar (Oxoid) and incubated aerobically at 37^0C for 16-24 hours, following which identification using the VITEK2®system (bioMérieux, Lyon, France) and susceptibility testing of colonies suggestive of *A. baumannii-calcoaceticus* complex was performed. All swabs, i.e. from patients, healthcare workers (HCWs), and environmental screening were processed in the same day.

Blood cultures were collected in BACTEC® (BD, Franklin Lakes, NJ, USA) bottles as per manufacturer's instructions at the discretion of attending clinicians with a minimum of 10 ml of blood collected from at least two puncture sites. Other clinical specimens were inoculated onto blood and MacConkey agar plates and incubated for 24 hours at 37°C. Subsequently, all colonies that had been cultured were examined for morphology by Gram stain and identified using the VITEK2®system.

Strains were stored in duplicate in -80°C in TSB with glycerol 10%. One tube of each strain was sent to the Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, the Netherlands, which laboratory holds an ISO 15189 accreditation, for further analysis. The other tube of each strain remained in the Indonesian laboratory. In the Netherlands, the identity of strains was confirmed using matrix-assisted laser desorption/ionisation (Maldi Biotyper, Bruker Microflex LT, London, UK).

The quality control strains used for this part of the study in Indonesia were *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, in the laboratory in Erasmus MC multiple quality control strains were used.

Antimicrobial Susceptibility Testing

The susceptibility of the screening isolates to imipenem and meropenem was investigated by standard Kirby-Bauer disc diffusion technique using Mueller-Hinton agar plates (BD). For the isolates from clinical cultures, approximation of the minimum inhibitory concentrations (MICs) of

antibiotics was determined by the VITEK2® system. Carbapenem zone sizes and MICs were interpreted according to EUCAST (2013) using the following breakpoints: meropenem zone size <21mm and MIC >2 mg/L, imipenem zone size <23 mm and MIC >2 mg/L.[7] For this part of the study, quality control strains as described above were used.

DNA Extraction and Carbapenemase Gene Detection

DNA from the isolates was extracted by a cell lysis step and boiling using the InstaGene Matrix (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. PCR-based detection of Ambler class B metallo-beta-lactamases (bla_{NDM}), class D beta-lactamases ($bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}51\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$) and IS*Aba1* were carried out using a T3000 Thermocycler (Biometra-Whatman, Goettingen). The upstream location of the IS*Aba1* insertion element of the blaOXA$_{\text{-}23\text{-}like}$ gene was demonstrated by using the IS*Aba1* forward primer and the $bla_{OXA\text{-}23\text{-}like}$ reverse primer. PCR primers and reaction conditions for PCR were as described previously [8-11]. Amplified PCR products were resolved by electrophoresis at 250 V for 30 minutes on 1.5% agarose gels with 0.5 x Tris (89mM)-boric acid (89mM)-EDTA(2mM) buffer containing SyBr® Safe DNA Gel Stain and visualized under UV light and photographed. In each run, a positive and negative control was included.

Clonal relatedness

Raman spectroscopy (SpectraCell RA® Bacterial Strain Analyzer, RiverD International BV, Rotterdam, The Netherlands) was applied as a first typing method. [12, 13] All isolates were grown overnight on trypticase soy agar (TSA; BD). Samples were prepared and submitted to spectrometry as described previously.[13] Raman light scatterings were analyzed by SpectraCell*RA* software version 1.9.0.13444:24. The similarity between pairs of spectra was calculated using the squared Pearson correlation coefficient (R^2-values), multiplied by 100 and expressed as a percentage. The similarity threshold for this study was set at 91% so that two isolates with an R^2 below this threshold were considered to be different and were designated different Raman types. Two isolates with an R^2-value above 99.5% were considered indistinguishable and were considered to have the same Raman type. In case of an R^2-value between of 90 % and 99.5%, these isolates were considered highly related but not identical. Correlation matrices displayed as 2D plots diagram were created using MATLAB version 7.1 (The MathWorks, Natick, MA, USA).

Multilocus sequence typing (MLST) was used as a second typing method for a subset of isolates, including isolates from the largest clones of Raman spectroscopy, and all isolates from blood cultures (one per patient). These isolates were subjected to whole genome sequencing

(WGS) using Illumina chemistry. MLST typing results were deduced from the WGS data and assigned based on the Oxford database (pubmlst.org/abaumannii).

Statistical Analysis

Statistical analyses were done using SPSS Version 24.0 (SPSS, Chicago, IL, USA). Patients admitted to adult ICU were compared to ER-ICU using Chi square or Fisher Exact and Mann-Whitney as appropriate. One-way ANOVA was used to compare patient characteristics according to their *A. baumannii-calcoaceticus* complex status. Univariate and multivariate analyses were performed to establish risk factors associated with mortality using a multivariate logistic regression model with backward selection and inclusion of variables with a p-value <0.1 in the univariate analysis. Cox proportional regression was used to analyse risk factors for length of stay. Kaplan-Meier method was performed to construct survival curves. P-values of less than 0.01 were considered significant. [14]

RESULTS

Patient Characteristics

During the 11-month study period, 1,211 patients were hospitalized in the ICUs (Adult ICU: 863, ER-ICU: 348). Supplementary Table 2 shows baseline characteristics of patients in each ICU. Of the 412 included patients, 188 were admitted to the adult ICU and 224 to the ER-ICU. There were no significant differences in characteristics between patients in both ICUs, except that in the adult ICU most of the patients had been referred from another ward in the same hospital (Supplementary Table 2). Therefore, we analyzed the data from the ICUs both separately and pooled.

Overall, 158/412 (38.3%) patients had a positive culture with carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex, the remaining 254 patients were free from carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex on admission and remained so during their ICU stay. Sixty-nine patients (69/412; 16.7%) already carried carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex as revealed by screening cultures taken on the day of ICU admission, 89/343 (25.9%) patients who were initially culture-negative acquired carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex during their ICU stay (Supplementary Figure 1). Of the total of 158 patients with positive cultures, the positive cultures were obtained from screening specimens in only 80 patients, from clinical specimens in only 34 patients and from both screening and clinical samples in 44 patients. Interestingly, of the patients that were positive on ICU admission, 17 (24.6%) were admitted directly from the emergency unit. Six patients had one or more blood cultures with carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex, and three of them died on the ICU. The dynamics of acquisition of

carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex in the ICU is shown in Figure 1, 60% of patients that became positive for Carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex during their ICU stay did so in the first week of ICU stay. There were no differences in the dynamics of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex acquisition between the two ICUs (median acquisition day in adult ICU: 7, in ER-ICU: 6). The acquisition rate for carbapenem-nonsusceptible *A. baumanni-calcoaceticus* complex was 43 per 1,000 patient-days at risk overall, with an average of 43 per 1,000 patient-days in adult ICU and 43 per 1,000 patient-days in ER-ICU.

Patient outcomes were clearly associated with carbapenem-nonsusceptible *A. baumanni-calcoaceticus* complex status of patients. Patients who acquired carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex during their ICU stay had a significantly longer length of stay (median [interquartile range (IQR)]: 11 [5-18], adjusted hazard ratio [aHR]: 2.56 [99% confidence interval (CI): 1.76-3.70], p<0.001, supplementary table 4, particularly the group of patients

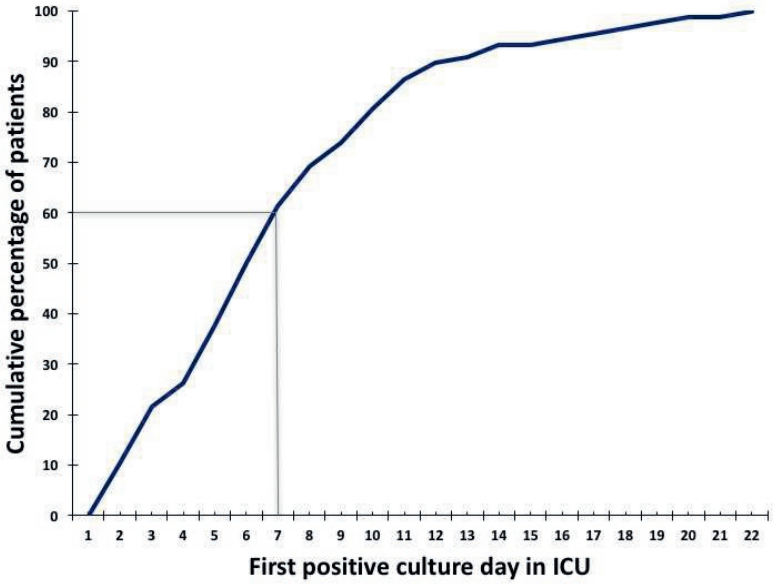

Figure 1. Acquisition of carbapenem-nonsusceptible Acinetobacter baumannii-calcoaceticus complex in ICUs. Note: The solid line represents the cumulative percentage of patients by first day of culture being positive for carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex during ICU stay. In total, data from 89 patients are included in this figure. The median acquisition day (day 7, 60% of patients positive) is shown as well.

that became positive before the day of their discharge (median [IQR] 13 [8-23] days, p<0.001, Figure 2) compared to the other groups of patients, of which ≥80% were discharged from the ICU

within ten days. Interestingly, these latter groups not only included the patients that were always free from carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex, but also included patients that already carried carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex at the time of admission to the ICU, and patients that remained free of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex until they were found to be positive by screening on the day of their discharge from the ICU (Figure 2).

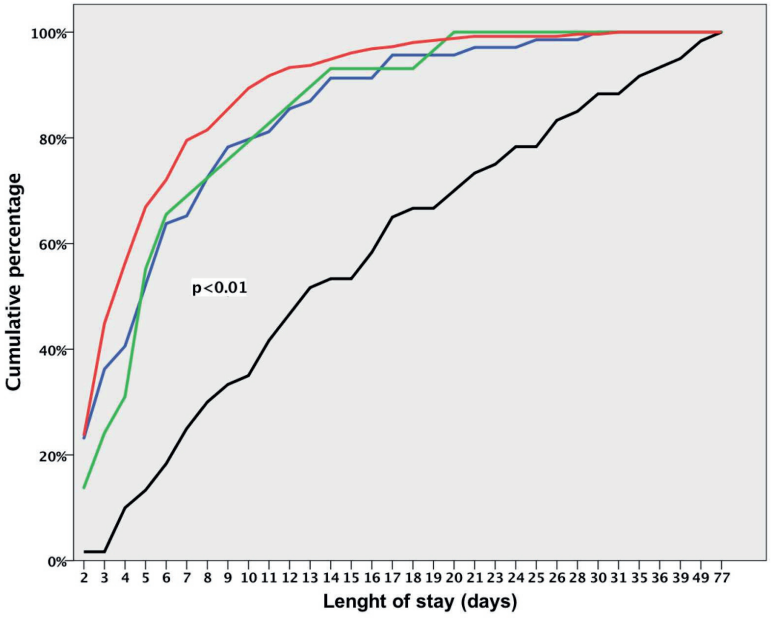

Figure 2. Cumulative percentage of length of stay for patients according to their carbapenem-nonsusceptible Acinetobacter baumannii-calcoaceticus complex status. Note: Lengths of stay (days) represent total days patients were hospitalized in the ICU. The red line represents patients that were always carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex negative during their ICU stay. The blue line represents patients already carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex positive on the day of admission. The green line represents patients that were carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex positive only at the time of discharge and the black line represents patients that became positive for carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex during their ICU stay before the day of discharge. P value: comparison between patients that became positive with carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex before the day of discharge and the other groups.

Acquisition of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex was not associated with mortality, 23.2% of patients that remained free of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex died versus 42.7% of patients that acquired carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex during their ICU stay (Figure 3,

Supplementary Table 3, for adjusted Odds Ratio (aOR):1.59 [99%CI: 0.74-3.40], p=0.066). Importantly, the admission SIRS and qSOFA scores of patients with or without *A. baumannii-calcoaceticus* complex acquisition did not differ (Table 1), indicating that the difference in the risk of dying was not present at the time of ICU admission but emerged later during their ICU stay (SIRS: crude Odds Ratio (cOR):1.69 [99%CI:0.55-5.22], p=0.230; qSOFA:cOR: 1.45[99%CI:0.68-3.08], p=0.211, Supplementary Table 3).

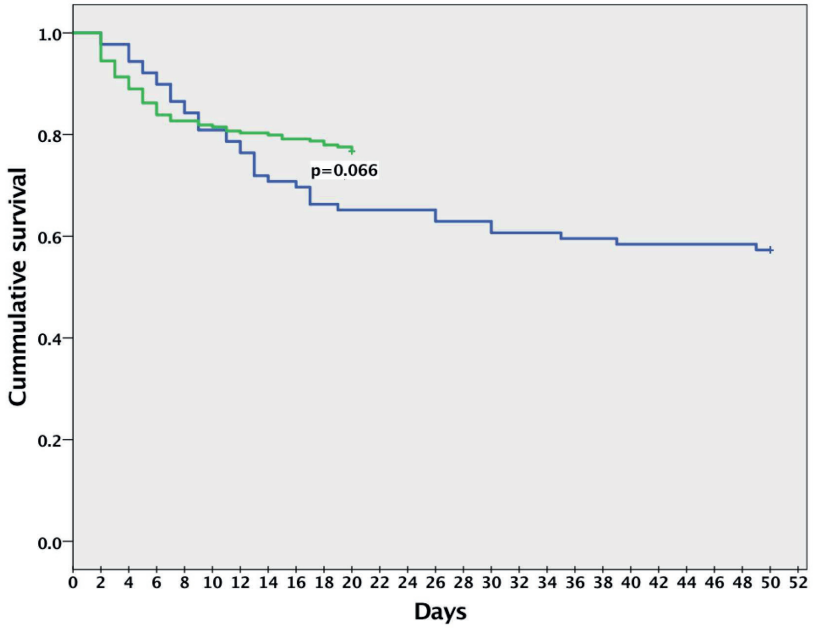

Figure 3. Survival analysis of ICU patients according to their carbapenem-nonsusceptible *Acinetobacter baumannii-calcoaceticus* complex status. Note: Survival of patients with carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex acquired during their ICU stay (blue line) compared with the survival of patients that remained negative for carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex in their screening and clinical cultures (green line).

Patients that were free of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex during their entire ICU stay were less likely to have had prior exposure to antibiotics, especially carbapenems (p<0.01), they were more likely to have had a surgical indication for their admission to the ICU, and less likely to have had cerebrovascular disease (Table 1). Patients that acquired carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex during ICU stay had undergone a procedure (mechanical ventilation), had medical device (central venous catheter or urine catheter) or had received carbapenem therapy more often than the other groups in the univariate analysis (p<0.01) (Table 1). In a multivariate comparison of patients who acquired carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex to patients that were always

negative only carbapenem therapy during ICU admission could be identified as a risk factor (aOR: 3.37 [99%CI: 1.68-6.77], p<0.01)

Carbapenem-nonsusceptible A. baumannii-calcoaceticus Complex and Molecular Characterization

In total, we collected 318 carbapenem-nonsusceptible isolated from 158 patients, six carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex isolates cultured from the environment (table, bed rails, sinks, and tapwater), and a single isolate from a healthcare worker (throat) that was carbapenem-nonsusceptible as well (Supplementary Table 5).

The *bla*$_{\text{OXA-23-like}}$ gene was demonstrated in 292/318 (91.8.0%) isolates including isolates from patients, the environment and from the healthcare worker. The *bla*$_{\text{OXA-24-like}}$ gene was detected in a single isolate. Coexistence of OXA-23 with other oxacillinases and carbapenemases was found: OXA-23/OXA-58 (1 isolate), and OXA-23/NDM-1 (4 isolates). The *bla*$_{\text{OXA-23}}$-like gene was always demonstrated in combination with the IS*Aba1* insertion element upstream to the OXA-23 β-lactamase. The intrinsic *A. baumannii-calcoaceticus* complex gene *bla*$_{\text{OXA-51}}$-like was demonstrated in all isolates. In the subset of isolates that were subjected to WGS (n=14), the *bla*$_{\text{OXA-51}}$-like gene involved *bla*$_{\text{OXA-66}}$ in 13 isolates and *bla*$_{\text{OXA-68}}$ in one isolate (Table 2).

Clonal relatedness

Raman spectroscopy analysis performed for all of the isolates, revealed the presence of multiple types within the collection of *A. baumannii-calcoaceticus* complex. In total, 51 Raman types were identified. Interestingly, the majority of strains belonged to one of five major clusters (Supplementary Figure 2). The largest cluster (designated CIPTO-31) consisted of 111 isolates obtained from 69 patients (screening and clinical specimens) and four isolates from the environment. The sources of the five major clusters are specified in Supplementary Table 4. Strains belonging to the dominant cluster CIPTO-31 were present in both ICUs throughout the study period, whereas other clones seemed to wax and wane over time (Figure 4). Patients were colonized with carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex irrespective of the location of their bed in these ICUs indicating that spreading of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex in the ICUs was not restricted to only a part of the ICU (Supplementary Figure 3).

MLST, performed for a subset of 14 isolates, revealed the presence of multiple sequence types (STs), which corresponded closely to the Raman spectroscopy clustering (Table 2). Four previously identified STs (ST195, ST208, ST218, and ST642) as well as several new STs, and a new allele for the *gpi* gene were found in this study (Table 2).

Table 1. Patient characteristics and outcomes according to Carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex status

	Carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex positive on admission	Carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex acquired during ICU stay	Carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex negative	p value
	n=69	(n=89)	n=254	
Age (years), median (IQR)	47 (33-60)	48 (35.3-57)	46 (32-58)	0.700
Gender (%)				0.535
Male	35 (50.7)	42 (47.2)	137 (53.9)	
Female	34 (49.3)	47 (52.8)	117 (46.1)	
Underlying Diseases (%)				
Cardiovascular				0.024
Yes	9 (13.0)	3 (3.4)	13 (5.1)	
No	60 (87.0)	86 (96.6)	241 (94.9)	
Cerebrovascular				0.001
Yes	3 (4.3)	14 (15.7)	12 (4.7)	
No	66 (95.7)	75 (84.3)	242 (95.3)	
Chronic Kidney Diseases				0.915
Yes	5 (7.2)	8 (9.0)	20 (7.9)	
No	64 (92.8)	81 (91.0)	234 (92.1)	
Diabetes Mellitus				0.334
Yes	20 (29.0)	20 (22.5)	78 (30.7)	
No	49 (71.0)	69 (77.5)	176 (69.3)	
Malignancy				0.740
Yes	29 (42.0)	37 (41.6)	116 (45.7)	
No	40 (58.0)	52 (58.4)	138 (54.3)	
Indication for ICU admission (%)				0.002

	Carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex positive on admission	Carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex acquired during ICU stay	Carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex negative	p value
	n=69	(n=89)	n=254	
Medical	32 (46.4)	38 (42.7)	70 (27.6)	
Surgical	37 (53.6)	51 (57.3)	184 (72.4)	
Referral from (%)				0.900
Other ward this hospital	38 (55.1)	48 (53.9)	136 (53.5)	
Other hospital	14 (20.3)	14 (15.7)	49 (19.3)	
Directly from Emergency Unit	17 (24.6)	27 (30.3)	69 (27.2)	
Antibiotic exposure (pre-ICU admission)				
Any antibiotic (%)	58 (84.1)	73 (82.0)	180 (70.9)	0.021
Carbapenem (%)	24 (34.8)	22 (24.7)	33 (13.0)	<0.01
SIRS Score, (%)				0.916
Score ≥2	64 (92.8)	81 (91.0)	232 (91.3)	
Score <2	5 (7.2)	8 (9.0)	22 (8.7)	
qSOFA Score, (%)				0.089
Score ≥2	51 (73.9)	78 (87.6)	205 (80.7)	
Score <2	18 (26.1)	11 (12.4)	49 (19.3)	
Procedures (during ICU admission)				
Mechanical ventilation (%)	63 (91.3)	88 (98.9)	220 (86.6)	0.004
Mechanical ventilation (days) median(IQR)	5 (2-8)	8 (4-16)	3 (1-6)	
≥5 days (%)	36 (52.2)	63 (70.8)	83 (2.7)	<0.01
<5 days (%)	33 (47.8)	26 (29.2)	171 (67.3)	
Central venous catheter (%)	66 (95.7)	85 (95.5)	212 (83.5)	<0.01
Central venous catheter (days) median(IQR)	6 (3-9)	10 (5-17)	4 (2-7)	

	Carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex positive on admission	Carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex acquired during ICU stay	Carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex negative	p value
	n=69	(n=89)	n=254	
≥5 days (%)	41 (59.4)	71 (79.8)	111 (3.7)	<0.01
<5 days (%)	28 (40.6)	18 (20.2)	143 (56.3)	
Urine catheter	69 (100)	89 (100)	254 (100)	N/A
Urine catheter (days) median (IQR)	6 (3-10)	10 (6-18)	5 (3-7)	
≥5 days (%)	26 (37.7)	13 (14.6)	122 (48.0)	<0.01
<5 days (%)	43 (62.3)	76 (85.4)	132 (52.0)	
Antibiotic therapy (during ICU admission)				
Any antibiotic (%)	68 (98.6)	89 (100)	249 (98.0)	0.411
Carbapenem (%)	42 (60.9)	62 (69.7)	95 (37.4)	<0.01
Outcomes				
Length of stay (days), median (IQR)	5 (3-9)	11 (5-18)	4 (3-7)	<0.01
Death	22 (31.9)	38 (42.7)	59 (23.2)	0.002

Abbreviations: ICU, Intensive Care Unit; IQR, Interquartile range; qSOFA, quick Sepsis-related Organ Failure Assessment; SIRS, Systemic Inflammatory Response Syndrome.

Significance was calculated using Oneway ANOVA, Pearson Chi Square and Fisher's Exact Test.

A p-value less than 0.01 was considered statistically significant.

**p<0.01

Table 2. Results of MLST analyses of 14 Carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex isolates.

Sample number	ST	gltA	gyrB	gdhB	recA	cpn60	gpi	rpoD	OXA-51 group	OXA-23	Raman cluster
171bl040813	new ST	1	15	3	2	2	164	3	OXA-66	+	CIPTO-31
262bl211013	new ST	1	15	3	2	2	164	3	OXA-66	+	CIPTO-31
275bl101013	new ST	1	15	3	2	2	61	3	OXA-66	+	CIPTO-31
69E-bed-rails-4	new ST	1	15	3	2	2	164	3	OXA-66	+	CIPTO-31
404re030714	195	1	3	3	2	2	96	3	OXA-66	+	CIPTO-48
91EIGD1214	195	1	3	3	2	2	96	3	OXA-66	+	CIPTO-48
156th250713	new allel/ST	1	3	3	2	2	new	3	OXA-66	+	CIPTO-48
206bl020913	new allel/ST	1	3	3	2	2	new	3	OXA-66	+	CIPTO-46
319bl020514	208	1	3	3	2	2	97	3	OXA-66	+	CIPTO-46
207re300813	new ST	1	3	3	2	2	61	3	OXA-66	+	CIPTO-45
422sp170714	new ST	1	3	3	2	2	61	3	OXA-66	+	CIPTO-45
116sp080713	218	1	3	3	2	2	102	3	OXA-66	+	CIPTO-30
176BA150813	218	1	3	3	2	2	102	3	OXA-66	+	CIPTO-30
153bl290713	642	22	15	13	12	4	169	2	OXA-68	+	CIPTO-39

Abbreviations: MLST, Multilocus Sequence Type; ST, Sequence Type.

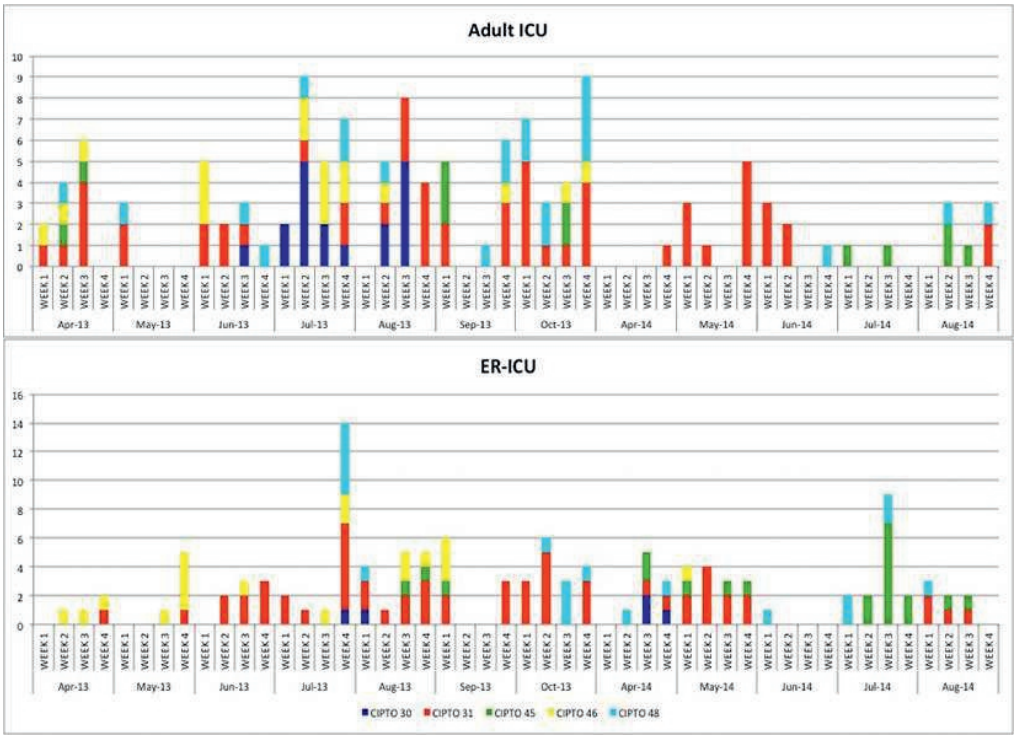

Figure 4. Persistence of Raman clones of carbapenem-nonsusceptible A*cinetobacter baumannii-calcoaceticus* complex in two ICUs of Dr. Cipto Mangunkusomo General Hospital, Jakarta, Indonesia. Note: Endemic curves of the five biggest clusters of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex in each ICU, April–October 2013 and April–August 2014. The dark blue bars represent cluster CIPTO-30. The red bars represent CIPTO-31. The green bars represent CIPTO-45. The yellow bars represent CIPTO-46 and the light blue bars represent CIPTO-48. The x-axis indicates time of the study (by week). The y-axis indicates number of isolates.

DISCUSSION

This is the first report of a study on the clinical and molecular epidemiology of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex in two ICUs in a large academic hospital in Jakarta, Indonesia. These two ICUs can be considered to have endemic carbapenem-nonsusceptible strains belonging to *A. baumannii-calcoaceticus* complex, i.e. entrenched by a few carbapenem-nonsusceptible clones, whose acquisition by patients may be associated with a prolonged ICU stay.

Carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex has emerged globally as a hospital-acquired pathogen, causing many outbreaks, especially in ICUs. [3] In Asia, carbapenem-resistant *A. baumannii-calcoaceticus* complex were found to dominate in Vietnam,

[15] Thailand, [16] Malaysia, [17] and also China. [18] Similar to these studies, we found that 38.3% of the patients had colonization or infection with carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex. By screening on ICU admission 16.7% of the carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex positive patients were already colonized with this species prior to their admission. This suggests that patients may become colonized with such strains elsewhere in the same hospital or in another hospital from which they are referred, or may come with such strain directly from the community, possibly having acquired their strain during a previous healthcare contact. Thus, the ICUs in this study experience a regular influx of patients carrying carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex strains into their setting. Our findings also raise questions about carriage of *A. baumannii-calcoaceticus* complex in the community, a finding that was also reported in a recent study from Semarang, Central Java, Indonesia. From the nasopharynx of 14 healthy people, *A. baumannii-calcoaceticus* complex was isolated in that study [19]. This requires further investigation.

Screening cultures can, therefore, be considered very helpful for early detection, infection control, and rational antibiotic use. A study in South Florida found that patients with positive surveillance cultures had a 8.4-fold higher risk of developing a subsequent *A. baumannii-calcoaceticus* complex infection compared with patients who remained negative on surveillance cultures.[20]

Our data also show that many patients acquire carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex during their ICU stay and that these acquisitions are associated with significantly longer ICU stay but not with mortality (at the chosen level of significance) compared to patients who did not acquire carbapenem-nonsusceptible *A. baumannii* while in the ICU. This is in agreement with a study from the USA, which showed an independent association between multidrug-resistant *A. baumannii-calcoaceticus* complex and increased hospital and ICU length of stay, but not an increased mortality. [21] However, a recent systematic review and meta-analysis to examine the association between carbapenem-resistant *A. baumannii-calcoaceticus* complex (CRAB) and mortality found that patients with CRAB had a significantly higher risk of mortality than patients with carbapenem-susceptible *A. baumannii-calcoaceticus* complex (crude OR = 2.22; 95% CI = 1.66- 2.98). [22]

The most prevalent mechanisms of carbapenem-nonsusceptibility in *A. baumannii-calcoaceticus* complex are acquired OXA-type carbapenem-hydrolyzing beta-lactamases of the OXA-23, OXA-24 and OXA-58 subfamilies, and the New Delhi metallo-beta-lactamases (NDM). [23-25] Our study found that 91.8% of the isolates carried the *bla*$_{\text{OXA-23}}$-like gene in combination with the upstream presence of the IS*Aba1* insertion element, enhancing carbapenem resistance. *bla*$_{\text{OXA-24}}$-like, *bla*$_{\text{OXA-58}}$-like, and *bla*$_{\text{NDM}}$-like genes were rarely present. The dissemination of OXA-23

producing carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex isolates has previously been reported in Asia and throughout the world. [26-28]

Carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex colonizing/infecting ICU patients may originate from the patient her/himself, but may also come from contaminated hospital equipment and environment, staff and other patients. Multiple reported outbreaks of multidrug-resistant *A. baumannii-calcoaceticus* complex infection were associated with environmental contamination. [29-31] There should be a focus on the prevention of nosocomial transmission of these microorganisms from these environmental sources to patients.

We performed Raman spectroscopy as a first bacterial typing method. [12] This analysis revealed five clusters, with the largest one (CIPTO-31), responsible for more than one third of all isolates, persisting in both ICUs throughout the study period. Geographical analysis of cluster CIPTO-31 isolates showed spreading of this clone throughout both ICUs. The isolates were found in and around all the beds regularly occupied by patients. MLST of four CIPTO-31 isolates revealed that these could be assigned to two new STs. Another nine isolates from the largest Raman clusters could be assigned to ST195, ST208, or ST218, or a new ST based on a new allele for the *gpi* gene (http://pubmlst.org/abaumannii/). A blood culture isolate that was unique in the Raman spectroscopy typing belonged to ST642. ST195, ST208, ST218, and ST642 have all previously been identified in Asian countries [32], including China [33], Malaysia [34], and Japan [35]. The epidemiology of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex in this Indonesian hospital was a combination of several known dominant Asian clones and new clones.

Our study has certain limitations. First, our study was a single-center study during a situation of endemic carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex colonisation and infection. Therefor, our data should not representative for the whole country. Second, we did not evaluate the effect of other possible confounders, such as dialysis, need for inotropes, surgery, and previous admission to a hospital.

CONCLUSIONS

In summary, this study is the largest to date that describes the characteristics and outcome of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex in ICUs of a referral hospital in Indonesia. Colonization or infection with carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex during hospitalization was independently associated with prolonged LOS in the ICU. Prevention of *A. baumannii-calcoaceticus* complex colonization and infection requires interventions directed to source control and limiting the transmission of such strains to and between patients.

DECLARATIONS

Ethics and Regulatory Considerations

The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the research on 17th September 2012, No: 561/PT02.FK/ETIK/2012, (No: 757/UN2.F1/ETIK/X/2014).

A Material Transfer Agreement (MTA) was reviewed and approved by the Director of National Institute Research and Development, Ministry of Health (No: LB.02.01/I.9.4/8500/2013).

Trial Registration: The study was retrospectively registered at www.trialregister.nl (No: NTR5541). Candidate number: 23527, NTR number: NTR5541, Date registered NTR: December 22nd,2015.

Informed Consent

Written informed consent was obtained using a form that was approved by the Ethics Committee Faculty of Medicine Universitas Indonesia/Dr.Cipto Mangunkusumo General Hospital. A signature and the date of signature was put on the form by the study subjects or their guardians and by the person who conducted the informed consent discussion and two witnesess. The signature confirmed that the consent was based on information that had been understood including publication.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Transparency Declaration

Yulia Rosa Saharman is an awardee of The DIKTI-NESO Scholarship by The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, and Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands.

Preliminary results of this study were presented at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) 2014 in Washington, DC (poster C-1477).

All authors report no conflict of interest relevant to this article.

Funding

This work was supported by 'The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia' and 'Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands'.

Authors' contributions

YRS, AK, PS, HAV, and JAS conceived the study and participated in design of the study. YRS, RS, and DA participated in acquisition of data.

YRS, WHFG, CHWK, HAV, and JAS performed data analysis and interpreted the data.

YRS, HAV, and JAS drafted the article.

All authors participated in critically revising the draft.

All authors read and approved the final manuscript.

Acknowledgements

We are thankful to The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, Dean of Faculty of Medicine Universitas Indonesia, Board of Directors of Dr. Cipto Mangunkusumo National General Hospital Jakarta Indonesia, Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands and Critical Care Division, Department of Anesthesia and Intensive Care, Faculty of Medicine Universitas Indonesia / Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia. We are grateful to Diana Willemse-Erix for helping us with the analysis of the Raman spectroscopy data, and dr. Ahmad Fuady for assistance with the statistical analysis.

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SUPPLEMENTAL MATERIALS

Supplementary Table 1. List of environmental samples

Sample site	Number of samples	
	Adult ICU	ER-ICU
Washbasin on ICUs ward	10	5
Monitor	14	10
Ventilator	15	5
Ambu bag	8	
Stethoscope	10	9
Drawer handle bedside cabinet	21	
Plastic multi-purpose container next to each bed	18	8
Stainless steel container	8	15
Flowmeter	15	
Infusion stand	11	8
Infusion pump	9	6
Bed rails	20	14
Tap water (washbasin on ICU ward)	10	8
Chart paper on bedside cabinet	11	6
Bedside cabinet table	15	11
Cleaning room: washbasin	9	
Cleaning room: sink countertop	3	
Cleaning room: mug	2	
Cleaning room: dish rack	3	
Mattress	5	4
Comb	3	
Water from siphon of washbasin	10	5
Water from mug next to each bed	10	6
Massage oil	5	
Chlorine solution after use	3	
Cleaning wipes	3	4
Wall	3	2
Drawer of bedside cabinet	3	
Water from suction	7	
Suction connector/container	3	
Water from humidifier	1	
Water after cleaning a floor	2	
Floor	2	
Nurse station	1	1

Abbreviations: ICU,Intensive Care Unit.

Supplementary Table 2. Baseline characteristics of patients admitted to the adult and Emergency Room (ER) ICUs

	Adult ICU	ER-ICU	p value
Number of patients enrolled	188	224	
Age (years), median (IQR)	49 (38-58)	43 (30-58)	0.041
Gender			
Male (%)	91 (48.4)	123 (54.9)	0.188
Female (%)	97 (51.6)	101 (45.1)	
Underlying diseases			
Cardiovascular (%)	12 (6.4)	13 (5.8)	0.806
Cerebrovascular (%)	10 (5.3)	19 (8.5)	0.211
Chronic kidney disease (%)	15 (8)	18 (8.0)	0.983
Diabetes mellitus (%)	80 (42.6)	38 (17.0)	0.000**
Malignancy (%)	62 (32.9)	120 (53.6)	0.000**
Indication for ICU admission			0.039
Medical (%)	54 (28.7)	86 (38.4)	
Surgical (%)	134 (71.3)	138 (61.6)	
Referral from			0.000**
Other ward this hospital (%)	144 (76.6)	78 (34.8)	
Other hospital (%)	20 (10.6)	57 (25.4)	
Directly from Emergency Unit (%)	24 (12.8)	89 (39.8)	
Antibiotic exposure (before admission to ICU)			
Any antibiotic (%)	146 (77.7)	165 (73.7)	0.349
Carbapenem (%)	40 (21.3)	39 (17.4)	0.321
SIRS Score, (%)			0.992
Score ≥2	172 (91.5)	205 (91.5)	
Score <2	16 (8.5)	19 (8.5)	
qSOFA Score, (%)			0.158
Score ≥2	158 (84.0)	176 (78.6)	
Score <2	30 (16.0)	48 (21.4)	
Procedures (during ICU admission)			
Mechanical ventilation (%)	170 (90.4)	201 (89.7)	0.815
Mechanical ventilation (days), median (IQR)	4 (1.5-9)	3 (2-7)	0.591
≥5 days (%)	87 (26.3)	95 (42.4)	0.431
<5 days (%)	101 (53.7)	129 (57.6)	
Central venous catheter (%)	166 (88.3)	197 (87.9)	0.913
Central venous catheter (days), median (IQR)	5.5 (3-10)	5 (3-8.5)	0.150
≥5 days (%)	106 (56.4)	117 (52.2)	0.400
<5 days (%)	82 (43.6)	107 (47.8)	
Urine catheter (%)	188 (100)	224 (100)	N/A
Urine catheter (days), median (IQR)	6 (3-11)	5 (3-9)	0.181
≥5 days (%)	118 (62.8)	133 (59.4)	0.486

	Adult ICU	ER-ICU	p value
<5 days (%)	70 (37.2)	91 (40.6)	
Antibiotic therapy (during ICU admission)			
Any antibiotic (%)	188 (100)	218 (97.3)	0.034
Carbapenem (%)	99 (52.7)	100 (44.6)	0.105
Outcomes			
Length of stay (days), median (IQR)	5 (3-10.75)	5 (3-8)	0.024
Death (%)	52 (27.7)	67 (29.9)	0.616

Abbreviations: ER-ICU, Emergency room Intensive Care Unit; ICU, Intensive Care Unit; IQR, Interquartile range; qSOFA, quick Sepsis-related Organ Failure Assessment; SIRS, Systemic Inflammatory Response Syndrome.

**p<0.01

Supplementary Table 3. Variables associated with mortality among patients with and without carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex

Variable	Mortality		p	Univariate analysis			p	Multivariate analysis		
					99% CI				99% CI	
	Death	%		cOR	Lower	Upper		aOR	Lower	Upper
Group										
Carbapenem-NS *A. baumannii-calcoaceticus complex* negative	59	49.6		1.00						
Carbapenem-NS *A. baumannii-calcoaceticus complex* positive on admission	22	18.5	0.143	1.55	0.72	3.33	0.862	0.95	0.41	2.19
Carbapenem-NS *A. baumannii-calcoaceticus complex* positive acquired	38	31.9	<0.01	2.46	1.26	4.82	0.119	1.59	0.74	3.40
Gender										
Male	66	55.5	0.362	1.22	0.70	2.14				
Female	53	44.5		1.00						
Underlying diseases										
Cardiovascular										
Yes	9	7.6	0.418	1.42	0.30	4.10				
No	110	92.4		1.00						
Cerebrovascular										
Yes	13	28.9	0.049	2.12	0.78	5.81	0.708	0.84	0.26	2.75
No	106	89.1		1.00				1.00		
Chronic kidney diseases										
Yes	11	9.2	0.556	1.25	0.46	3.40				
No	108	90.8		1.00						
Diabetes mellitus										
Yes	30	25.4	0.327	0.79	0.42	1.48				
No	89	74.8		1.00						
Malignancy										
Yes	43	36.1	0.036	0.63	0.35	1.12	0.294	0.75	0.37	1.52
No	76	63.9		1.00				1.00		
Indication for ICU admission										
Medical	61	51.3	<0.01	2.85	1.59	5.10	<0.01	1.98	1.07	3.68
Surgical	58	48.7		1.00				1.00		
Referral from										
Other ward this hospital	58	48.7	0.798	1.69	0.75	3.80	0.171	1.53	0.67	3.50
Other hospital	30	25.2	0.096	0.94	0.48	1.83	0.182	1.50	0.70	3.19
Directly from Emergency Unit	31	26.1		1.00				1.00		
Antibiotic exposure (before admission to ICU)										

Variable	Mortality		p	Univariate analysis			p	Multivariate analysis		
					99% CI				99% CI	
	Death	%		cOR	Lower	Upper		aOR	Lower	Upper
Any antibiotic	100	84.0	0.011	2.05	0.99	4.23	0.264	1.44	0.62	3.31
No any antibiotic	19	16.0		1.00				1.00		
Carbapenem	36	30.3	<0.01	2.52	1.29	4.91	0.111	1.61	0.75	3.46
No carbapenem	83	69.7		1.00				1.00		
SIRS score										
Score ≥2	112	94.1		1.00						
Score <2	7	5.9	0.230	1.69	0.55	5.22				
Q Sofa										
Score ≥2	101	84.9		1.00						
Score <2	18	15.1	0.211	1.45	0.68	3.08				
Procedures (during ICU admission)										
Mechanical ventilation used (%)	119	100.0	N/A							
No mechanical ventilation used (%)	0	0.0								
Mechanical Ventilation (days)										
≥5 days	74	62.2	<0.01	2.82	1.58	5.02	<0.01	4.71	1.36	16.30
<5 days	45	37.8		1.00				1.00		
Central venous catheter use	116	97.5	<0.01	7.20	1.51	34.34	<0.01	5.76	1.06	31.20
No central venous catheter used	3	2.5		1.00				1.00		
Central Venous Catheter (days)										
≥5 days	77	64.7	0.006	1.85	1.04	3.29	0.011	3.59	0.99	13.05
<5 days	42	35.3		1.00				1.00		
Urine catheter used	119	100.0	N/A							
No urine catheter used	0	0.0								
Urine Catheter (days)										
≥5 days	85	71.4	0.005	1.91	1.05	3.50	0.647	1.29	0.31	5.26
<5 days	34	28.6		1.00				1.00		
Antibiotic therapy (during ICU admission)										
Any antibiotic	119	100.0	N/A							
No any antibiotic	0	0.0		1.00						
Carbapenem	81	68.1	<0.01	3.16	1.75	5.72	0.002	2.17	1.13	4.16
No carbapenem	38	31.9		1.00				1.00		
Age, median (IQR) (years old)	50	(38-60)	0.009	1.02	1.00	1.04	0.166	1.01	0.99	1.03
LOS, median (IQR) (days)	6	(3-12)	0.058	1.03	0.99	1.06				

Abbreviation: aOR, adjusted Odds Ratio; cOR, crude Odds Ratio; CI, Confidence Interval; ICU: Intensive Care Unit; IQR, Interquartile range; LOS, Length of Stay; NS, Nonsusceptible; SIRS, Systemic Inflammatory Response Syndrome; qSOFA, quick Sepsis-related Organ Failure Assessment.

Supplementary Table 4. Variables associated with length of stay among patients with and without carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex

Variable	Univariate analysis					Multivariate analysis			
	Length of stay	p	cHR	99% CI		p	aHR	99% CI	
	Median (IQR)			Lower	Upper			Lower	Upper
Group									
Carbapenem-NS *A. baumannii-calcoaceticus complex* negative	4 (3-7)		1.00				1.00		
Carbapenem-NS *A. baumannii-calcoaceticus complex* positive on admission	5 (3-9)	0.045	1.31	0.93	1.87	0.755	1.04	0.73	1.50
Carbapenem-NS *A. baumannii-calcoaceticus complex* positive, acquired	11 (5-18)	<0.01	2.73	1.93	3.88	<0.01	2.56	1.76	3.70
Gender									
Male	5 (3-10)	0.426	0.92	0.72	1.19				
Female	4 (3-9)		1.00						
Underlying disease									
Cardiovascular									
Yes	8 (5-12)	0.199	1.31	0.77	2.22				
No	5 (3-9)		1.00						
Cerebrovascular									
Yes	8 (3-14)	0.026	1.54	0.93	2.55	0.580	0.89	0.52	1.53
No	5 (3-9)		1.00				1.00		
Chronic kidney disease									
Yes	7 (4-14)	0.108	1.34	0.84	2.14				
No	5 (3-9)		1.00						
Diabetes mellitus									
Yes	4 (3-9)	0.214	0.87	0.66	1.16				
No	5 (3-9)		1.00						
Malignancy									
Yes	5 (3-8)	0.254	0.89	0.69	1.15				
No	5 (3-10)		1.00						
Indication for ICU admission									
Medical	6 (3-12)	<0.01	1.52	1.15	2.01	0.599	1.06	0.79	1.44
Surgical	4 (3-8)		1.00				1.00		
Referral from									
Other ward this hospital	5 (3-10)	0.151	1.18	0.88	1.59				

Variable	Univariate analysis					Multivariate analysis			
	Length of stay	p	cHR	99% CI		p	aHR	99% CI	
	Median (IQR)			Lower	Upper			Lower	Upper
Other hospital	5 (3-9)	0.197	1.21	0.83	1.77				
Directly from Emergency Unit	5 (3-8)		1.00						
Antibiotic exposure (before admission to ICU)									
Any antibiotic	5 (3-10)	0.014	1.33	0.99	1.79	0.037	1.28	0.94	1.74
No antibiotic	4 (3-7)		1.00				1.00		
Carbapenem	8 (3-13)	0.012	1.37	0.99	1.90	0.168	1.22	0.84	1.75
No carbapenem	5 (3-8)		1.00				1.00		
SIRS score									
Score ≥2	5 (3-9)	0.651	1.08	0.69	1.71	0.255	1.23	0.77	1.99
Score <2	4 (2-7)		1.00				1.00		
qSOFA									
Score ≥2	5 (3-10)	<0.01	1.62	1.17	2.25	0.112	1.24	0.88	1.74
Score <2	3 (2-6)		1.00				1.00		
Procedures (during ICU admission)									
Mechanical ventilation	5 (3-10)	<0.01	2.50	1.61	3.89	0.659	1.09	0.67	1.77
No mechanical ventilation	3 (2-4)		1.00				1.00		
Mechanical Ventilation (days)									
≥5 days	10 (7-15)	<0.01	6.33	4.592	8.727	<0.01	3.10	2.00	4.79
<5 days	3 (2-4)		1.00				1.00		
Central venous catheter	5 (3-10)	<0.01	2.12	1.42	3.17	0.462	0.87	0.53	1.43
No central venous catheter	3 (2-5)		1.00				1.00		
Central venous catheter (days)									
≥5 days	9 (6-13)	<0.01	6.30	4.62	8.59	0.009	1.77	1.01	3.08
<5 days	3 (2-3)		1.00				1.00		
Urine catheter	5 (3-9)	N/A							
No urine catheter	N/A								
Urine catheter (days)									
≥5 days	8 (5-12)	<0.01	9.87	6.84	14.24	<0.01	3.26	1.85	5.74
<5 days	3 (2-3)		1.00				1.00		
Antibiotic therapy (during ICU admission)									
Any antibiotic	5 (3-9)	0.005	3.20	1.10	9.33	0.344	1.49	0.50	4.43
No antibiotic	2 (2-3)		1.00				1.00		
Carbapenem	7 (4-12)	<0.01	1.78	1.37	2.32	0.570	0.93	0.68	1.28
No carbapenem	4 (2-7)						1.00		

Variable	Univariate analysis					Multivariate analysis			
	Length of stay	p	cHR	99% CI		p	aHR	99% CI	
	Median (IQR)			Lower	Upper			Lower	Upper
Age, correlation coefficient (r)	0.081	0.154	1.00	0.99	1.00				
Mortality	6 (3-12)	0.057	1.23	0.93	1.63	0.016	0.76	0.57	1.02

Abbreviation: aHR, adjusted Hazard Ratio; cHR, crude Hazard Ratio; CI, Confidence Interval, ICU: Intensive Care Unit; IQR, Interquartile Range; LOS, Length of Stay; NS, Nonsusceptible; SIRS, Systemic Inflammatory Response Syndrome; qSOFA, quick Sepsis-related Organ Failure Assessment .

Supplementary Table 5. Source of detection of the carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex isolates collected in the study

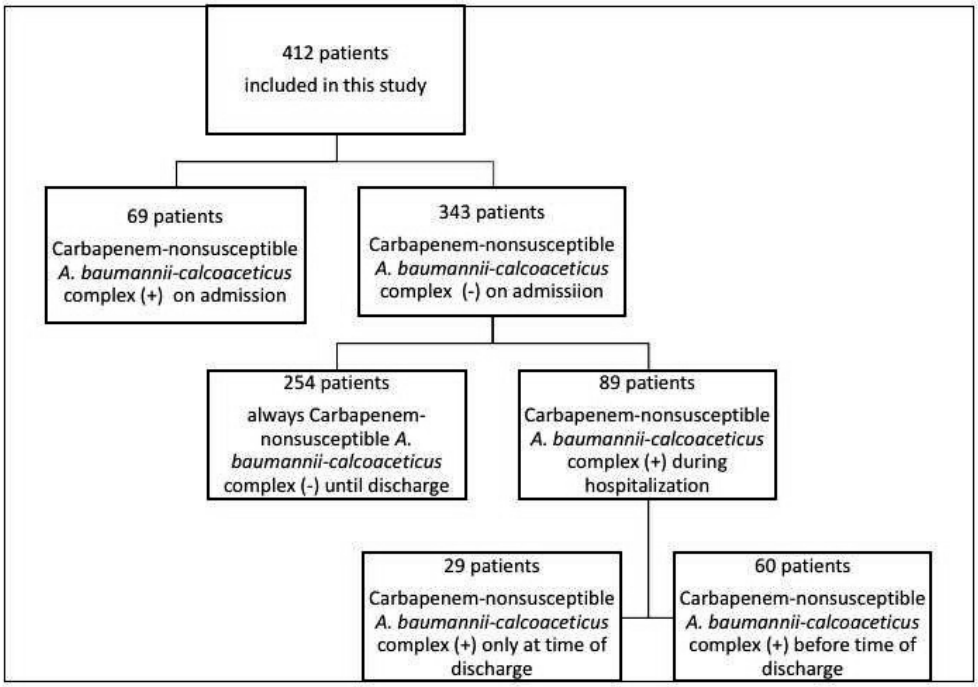
Culture	Number of patients	Isolates
Only screening	80	131
Only clinical specimen	34	49
Screening and clinical specimen	44	131
Environment		6
Healthcare worker screening		1
Total	**158**	**318**

Supplementary Table 6. Sources of the five major Raman clusters of carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex in adult ICU and ER-ICUs

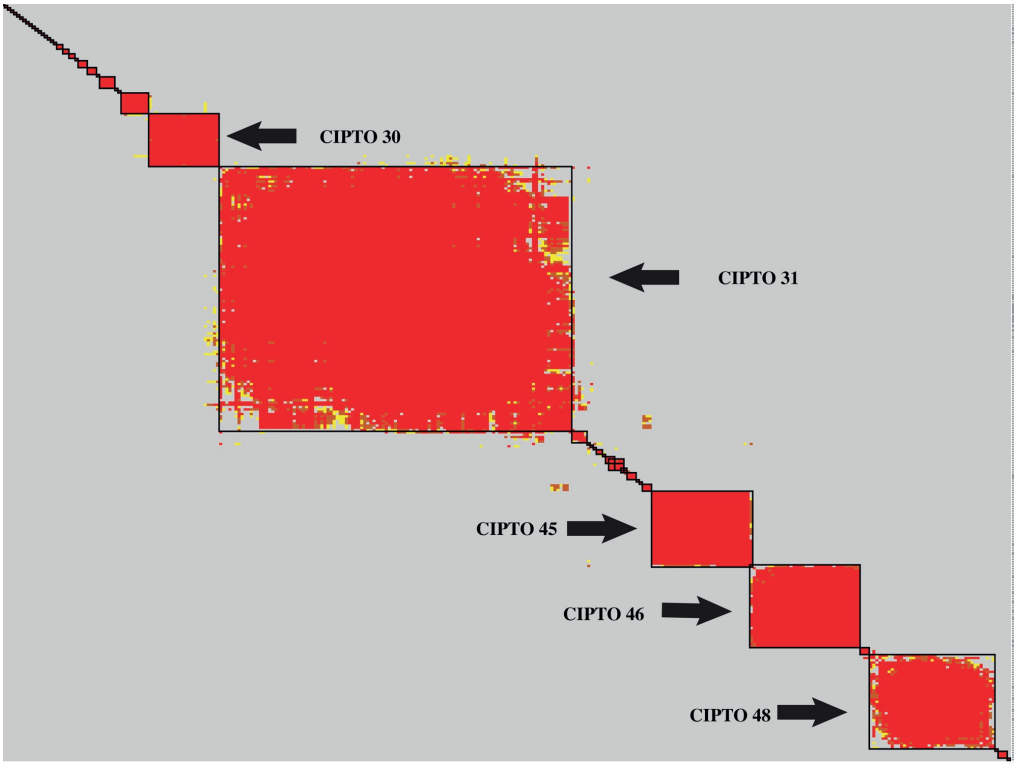
Raman cluster	Number of isolates (patients)	Origin of strain					
		Patients				Environment	
		Adult ICU		ER-ICU		Adult ICU	ER-ICU
		Screening	Clinical	Screening	Clinical		
CIPTO-30	23 (14)	12	6	4	1		
CIPTO-31	111 (69)	35	17	33	26	4	
CIPTO-45	33 (19)	8	4	17	4		
CIPTO-46	36 (27)	7	10	10	9		
CIPTO-48	40 (29)	12	9	14	5	1	

Abbreviation: ER-ICU, Emergency room Intensive Care Unit; ICU, Intensive Care Unit.

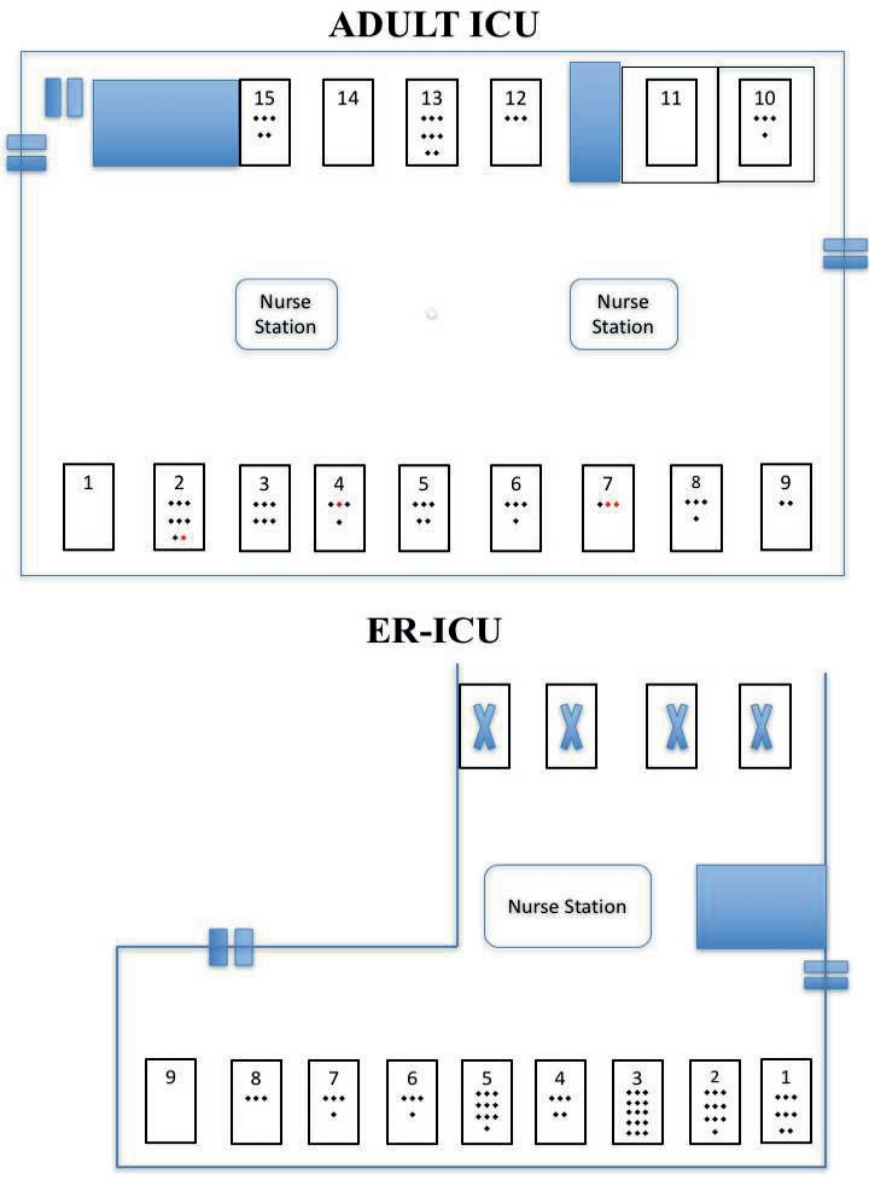
Five largest Raman clusters are indicated by CIPTO-30, CIPTO-31, CIPTO-45, CIPTO-46, and CIPTO-48.



Supplementary Figure 1. Carbapenem-nonsusceptible *Acinetobacter baumannii-calcoaceticus* complex carriage of included patients admitted to adult and ER-ICUs of Dr. Cipto Mangunkusomo General Hospital, Jakarta, Indonesia.



Supplementary Figure 2. Raman spectroscopy-based cluster analysis of *Acinetobacter baumannii-calcoaceticus* complex isolates from adult and ER-ICUs. Note: Raman spectra correlation matrix of carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex isolates. Isolates are shown in a color-scale (red-orange-yellow-grey) based on their similarity of correlation coefficient value. Red clusters (91–100%) indicate isolates that are indistinguishable according to the cut-off value. Grey areas (≤70%) indicate isolates that are not related. The potentially related isolates are shown by yellow areas (lower similarities (71–80%)) and orange areas (higher similarities (81–90%)).



Supplementary Figure 3. The bed-clone analysis of cluster CIPTO-31 carbapenem-nonsusceptible *Acinetobacter baumannii-calcoaceticus* complex. Note: The bed-clone analysis from cluster CIPTO-31 carbapenem-nonsusceptible *A. baumannii-calcoaceticus* complex showed spreading of 115 isolates in both ICUs. The isolates were found in patients from almost all the beds. A red diamond represents an environmental isolate.

CHAPTER 4

4

Clinical Impact of Endemic NDM-producing *Klebsiella pneumoniae* in Intensive Care Units of the National Referral Hospital in Jakarta, Indonesia

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ABSTRACT

Objective:

A prospective observational study was performed to assess the epidemiology and clinical impact of carbapenem-non-susceptible *Klebsiella pneumoniae* (CNKP) in intensive care units (ICUs) of the national referral hospital in Jakarta, Indonesia.

Materials/methods:

Adult patients consecutively hospitalized for >48 hours in two ICUs of the national referral hospital were included from April until October 2013 and from April until August 2014. *K. pneumoniae* from clinical cultures and standardized screening of rectum and throat on admission, discharge and weekly if hospitalized >7 days were collected. Environmental niches and healthcare workers (HCWs) were also screened. Susceptibility was determined phenotypically and the presence of carbapenemase genes by PCR. Raman spectroscopy as well as multiple-locus variable number tandem repeat analysis (MLVA) were used for typing.

Results:

Twenty-two out of 412 (5.3%) patients carried CNKP on admission and 37/390 (9.5%) acquired CNKP during ICU stay. The acquisition rate was 24.7/1,000 patient-days at risk. One out of 31 (3.2%) environmental isolates was a CNKP. None of the HCWs carried CNKP. Acquisition of CNKP was associated with longer ICU stay (adjusted hazard ratio: 2.32 [CI_{99}: 1.35-3.68]). ICU survival was lower among patients with CNKP compared to patients with carbapenem-susceptible *K. pneumoniae* (aHR 2.57, p=0.005). Ninety-six of the 100 (96%) CNKP isolates carried a carbapenemase gene, predominantly *bla*$_{NDM}$. Raman typing revealed three major clusters among 48 Raman types identified, whereas MLVA distinguished six major clusters among a total of 30 different genotypes.

Conclusions:

NDM-producing CNKP are introduced into these ICUs and some strains expand clonally among patients and the environment, resulting in endemic CNKP. CNKP acquisition was associated with prolonged ICU stay and may affect ICU survival.

Trial registration:

The study was registered at Netherlands Trial Register *http://*www.trialregister.nl (No: 5541). Candidate number: 23527, NTR number: NTR5541, Retrospectively registered: NTR: 22 December 2015.

Keywords:

Klebsiella pneumoniae, microbial drug resistance, carbapenemase, intensive care unit, mortality, Indonesia.

INTRODUCTION

Carbapenems are the antibiotics of choice for treatment of life-threatening infections due to multidrug-resistant Gram-negative bacilli. However, the worldwide emergence of carbapenem-non-susceptible *Klebsiella pneumoniae*, especially in intensive care units (ICUs), has become a major challenge. Non-susceptibility to carbapenems in *K. pneumoniae* may be due to production of Ambler class A β-lactamases (e.g. KPC), class B metallo-β-lactamases (MBLs, e.g. VIM, IMP, NDM) or class D oxacillinases (e.g. OXA-48 like enzymes).(1-3)

Although carbapenemase-producing *K. pneumoniae* have emerged globally, geographic variations do exist. *K. pneumoniae* producing KPCs have initially mainly been reported in the USA and Israel, but more recently also from China and Taiwan.(1, 2, 4, 5) *K. pneumoniae* strains carrying OXA-48-like carbapenemases were first described in Turkey in 2003. Currently, *K. pneumoniae* with OXA-48-like carbapenemases are spreading rapidly in many European countries, in addition to being endemic in the Middle East and in Northern Africa. (1, 4, 5) Bacteria with the New Delhi metallo-β-lactamase (NDM) enzyme, which was first identified in Sweden from a patient who had travelled from New Delhi, India, have attained endemic levels in countries of the Indian subcontinent including India, Pakistan, Bangladesh and Sri Lanka.(1, 4-8) This gene is also encountered in bacteria, including *K. pneumoniae*, in some countries in the South East Asian region, including Singapore (9), Thailand (10) and Vietnam (1). However, so far there have been few data on the epidemiology of carbapenem-non-susceptible *K. pneumoniae* reported from Indonesia, the fourth most populous country in the world. In 2011, 27.6% of the Enterobacteriaceae isolated from specimens at two ICUs in Jakarta was carbapenem-resistant, including one *K. pneumoniae* harboring the bla_{NDM} gene.(11) In 2014-2015, the prevalence of resistance to meropenem among *K. pneumoniae* from urinary tract infections in clinical and outpatient clinical settings was 21.6%, but no further analysis of these isolates was performed. (12) The aim of the present study was to delineate the clinical and molecular epidemiology of carbapenem-non-susceptible *K. pneumoniae* isolated in two ICUs of the Dr. Cipto Mangunkusumo General Hospital, the national referral teaching hospital in Jakarta.

MATERIALS AND METHODS

Study design

A prospective observational study was performed in a 1,000-bed national referral teaching hospital with 34,000 admissions per year in Jakarta, Indonesia, from April until October 2013 and from April until August 2014. Two ICUs participated, the adult ICU and the Emergency Room (ER)-ICU, with 865 and 390 admissions in 2013, respectively, and 1,154 and 439

admissions in 2014, respectively. The adult ICU is a 12-bed open ward with mechanical ventilation facilities, admitting patients with various medical and surgical indications, and one designated nurse per patient during morning shifts and a 1:1.5 nurse/patient ratio during other shifts. It is also used as post-anaesthetic care unit. The ER-ICU has the same design, but 8 beds, and the nurse per patient ratio in the morning shifts is 1:1 and during the other shifts 1:2. This ICU is also used for short observations.

All adult patients (≥18 years old) admitted to one of the two ICUs and hospitalized for more than 48 hours were eligible for enrollment in this study. Informed consent was obtained from the patient or their relatives as applicable. Demographic and clinical characteristics such as age, gender, medical or surgical indication, underlying diseases, hospitalization history, and previous use of antibiotics on admission were recorded.

Systemic inflammatory response syndrome (SIRS) criteria on admission were used as a screening tool to assess (severity of) septic illness. The "Acute Physiology and Chronic Health Evaluation II" score was not feasible in this low-resource setting. SIRS is defined as two or more of the following: fever >38°C or <36°C, heart rate >90 beats per minute, respiratory rate >20 breaths per minute or PaCO2 <32 mmHg, abnormal white blood cell count (>12,000/mm^3 or <4,000/mm^3 or >10% bands). (13)

The quick Sequential Organ Failure Assessment (qSOFA) score is a newer bedside prompt that may identify patients with suspected infection and helps to determine sepsis in all healthcare environments. The qSOFA score assigns one point for each of the following conditions: systolic blood pressure ≤100 mmHg, respiratory rate ≥22 breaths per minute, and altered mentation (Glasgow coma scale <15). The score ranges from 0 to 3 points. A qSOFA score ≥2 at the onset of infection is associated with a greater risk of death and prolonged ICU stay. This score was included as well. (13)

Acquisition is defined as a screening culture (throat or rectum/stool) or clinical culture with a first detection of *K. pneumoniae* with reduced susceptibility to a carbapenem, that was not present in cultures taken from the patient on admission or in the first 48 hours of admission. Outcome measures were acquisition of a carbapenem-susceptible and carbapenem-non-susceptible *K. pneumoniae* (independent of resistance to other classes of antibiotics), length of stay in the ICU, and mortality during ICU stay.

Environmental samples were taken twice (in October 2013 and December 2014), simultaneously in both ICUs. Screening of healthcare workers (HCWs) was performed once. HCWs were defined as all personnel including doctors, nurses and other people (cleaning staff, administration staff, porters, nutritionist) working in one of the two ICUs during the study period.

Sampling

From patients enrolled, screening cultures were obtained from throat and rectum or stools by experienced ICU nurses on the day of admission, at the time of discharge from the ICU, and weekly if the patient was admitted for seven days or more. The samples were collected with sterile cotton-tipped swabs and placed in Amies transport medium (Oxoid, Basingstoke, UK). The swabs were transported in clean, closed boxes at ambient temperature to the laboratory on the same day. All swabs were processed in the laboratory within 24 hours.

Clinical samples were collected from a patient when the ICU physician suspected the patient of having an infection. Specimens were taken under aseptic precautions from the lower respiratory tract, blood, urine, tissue, or wound, on indication.

Environmental samples were taken from various sites, including wash basins, bed rails, bedside cabinet tables, ventilators, and monitor screens (Supplementary Table 1), with sterile cotton-tipped swabs and placed in Amies transport medium.(14) All HCWs working in one of the ICUs were sampled (rectal and throat) once over the course of one month (September 2013) with sterile cotton-tipped swabs, which were transported to the laboratory in Amies transport medium.

Microbiological Methods

Isolation and identification of bacteria

In the laboratory, each screening swab was placed in a trypticase soy broth (TSB) supplemented with cefotaxime 2 mg/L plus vancomycin 50 mg/L and incubated overnight. The next day, a loop of broth was sub-cultured on MacConkey agar (Oxoid).

Blood cultures were collected in BACTEC (BD, Franklin Lakes, NJ, USA) bottles as per manufacturer's instructions with a minimum of 10 mL of blood collected from at least two puncture sites. Other clinical specimens were inoculated onto blood and MacConkey agar plates (Oxoid) and incubated for 24 hours at 37°C. All morphologically different colonies were examined by Gram stain and identified using the VITEK2® system (bioMérieux, Lyon, France).

Strains were stored in duplicate in -80°C in TSB with glycerol 10%. One tube of each strain was sent to the Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, the Netherlands, for further analysis. The other tube of each strain remained in the Indonesian laboratory. In the Netherlands, the identity of strains was confirmed using matrix-assisted laser desorption/ionisation (Maldi Biotyper, Bruker Microflex LT, Bruker, London, UK).

The quality control strains used for this part of the study in Indonesia were *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, in the laboratory of Erasmus MC multiple quality control strains were used.

Antimicrobial susceptibility testing

Imipenem and meropenem susceptibility tests on isolates from screening cultures were performed by standard Kirby-Bauer disc diffusion technique using Mueller-Hinton agar plates (BD) based on EUCAST Disc Diffusion Method for Antimicrobial Susceptibility Testing- Version 3.0 (April 2013). Minimum inhibitory concentrations (MICs) of antibiotics were determined by VITEK2® for clinical isolates. Carbapenem MICs and zone sizes were interpreted according to EUCAST (2013) using the following breakpoints for non-susceptibility: meropenem >0.25 mg/L (<24mm), imipenem >1 mg/L (<22 mm).(15) For this part of the study, quality control strains as described above were used.

String test

In order to determine hyper-muco-viscosity, the string assay was performed for all *K. pneumoniae* isolates. For this, the strains were inoculated onto 5% sheep blood agar (BD) and kept overnight at 37°C. An individual colony was then touched with a 1 µL disposable loop which was subsequently pulled up slowly. The string test was deemed positive if a string of ≥5 mm was formed between the colony and the loop.(16)

Phenotypic detection of carbapenemase

A phenotypic detection test for Ambler class A and B and OXA-48-like carbapenemases was performed with discs (Rosco Diagnostica A/S, Taastrup, Denmark) containing meropenem (10 µg), temocillin (30 µg), meropenem + phenyl boronic acid (PBA), meropenem + dipicolinic acid (DPA), meropenem + PBA + DPA, and meropenem + cloxacillin (CL), using a 0.5 McFarland suspension of the isolates on Mueller Hinton II agar plates. Zone diameters were measured after overnight incubation at 37°C. The temocillin zone diameter was only interpreted if no synergy was observed with DPA and/or PBA. Isolates without synergy with the PBA or DPA test and a temocillin zone diameter ≤10 mm (i.e. the absence of an inhibition zone around the temocillin disc) were considered OXA carbapenemase positive. The interpretation of the PBA and DPA synergy tests and the temocillin disc diffusion were as described previously.(17)

DNA extraction and PCR for carbapenemase genes

DNA from the isolates was extracted by a cell lysis step and boiling using the InstaGene Matrix (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. PCR-based detection of Ambler class A carbapenemases (bla_{KPC}), Ambler class B metallo-β-lactamases (bla_{NDM}), and class D β-lactamases ($bla_{OXA\text{-}48\text{-}like}$) were carried out using T3000 Thermocycler (Biometra-Whatman, Goettingen). PCR primers and reaction conditions for PCR were as described previously.(18-20) Amplified PCR products were resolved by electrophoresis at 250 V

for 30 minutes on 1.5% agarose gels with 0.5 x Tris (89mM)-boric acid (89mM)-EDTA (2mM) buffer containing SyBr® Safe DNA Gel Stain and visualized under UV light and photographed. In each run, a positive and negative control was included.

Clonal relatedness

Raman spectroscopy (SpectraCell RA® Bacterial Strain Analyzer, RiverD International BV, Rotterdam, The Netherlands) was applied as a first typing method.(21, 22) All isolates were grown overnight on trypticase soy agar (TSA; BD). Samples were prepared and submitted to spectrometry as described previously.(22) Raman light scatterings were analyzed by SpectraCell*RA* software version 1.9.0.13444:24 (RiverD). The similarity between pairs of spectra was calculated using the squared Pearson correlation coefficient (R^2-values), multiplied by 100 and expressed as a percentage. The similarity threshold for this study was set at 91% so that two isolates with an R^2 below this threshold were considered to be different and were designated different Raman types. Two isolates with an R^2-value above 99.5% were considered indistinguishable and were considered to have the same Raman type. In case of an R^2-value between of 91% and 99.5%, these isolates were considered highly related but not identical.(23) Correlation matrices displayed as 2D plots diagram were created using MATLAB version 7.1 (The MathWorks, Natick, MA, USA).

Multiple-locus variable number tandem repeat analysis (MLVA) was used as a second typing method. The MLVA typing protocol was based on Brink et al.(24) with minor modifications (for details, see Supplement). DNA was quantitated using PicoGreen dsDNA reagent (Invitrogen, Bleiswijk, The Netherlands). Amplification reactions contained approximately 1 ng of DNA and primers according to Supplementary Table 2 in 1x Roche FastStart PCR Master Mix (Roche diagnostics, Almere, The Netherlands). The thermocycling protocol consisted of an initial denaturation for 5 min at 95°C followed by 30 amplification cycles of denaturation for 30s at 95°C, 30s annealing at 58°C and 1 min extension at 72°C. A final extension step of 30 min at 72°C was applied before reactions were cooled to room temperature. Before loading, amplification products were diluted 100x, combined with the GeneScan 600 LIZ Dye Size Standard (ThermoFisher Scientific, Bleiswijk, The Netherlands) and run on an ABI 3130 capillary electrophoresis platform (ThermoFisher Scientific) using recommended conditions. Electropherograms were analyzed using the MLVA plugin in BioNumerics v7.6 software (Applied-Maths, Sint-Martens-Latem, Belgium). Assignment of repeat numbers was calibrated by comparing our results to those obtained with selected isolates that were genotyped by the Maastricht lab. Typing data was analyzed categorically.

Statistical analysis

Statistical analyses were performed using SPSS Version 24.0 (SPSS, Chicago, IL, USA). Baseline characteristics from patients admitted to the adult ICU were compared to those in the ER-ICU using Chi square and Mann-Whitney as appropriate. One-way ANOVA was used to compare patient characteristics according to their *K. pneumoniae* status. Univariate and multivariate analyses were performed to establish risk factors associated with in-ICU mortality using a multivariate logistic regression model with backward selection and inclusion of variables with a p value <0.1 in the univariate analysis. Cox proportional regression was used to analyze risk factors for length of stay. Kaplan-Meier method was performed to construct survival curves.The R-code (R-3.6.2.pkg binary for OS X 10.11 software can be obtained via CRAN, the Comprehensive R Archive Network, http://cran.R-project.org) was used to calculate the competing risks estimates (competing risk analysis is available in an add-on package called cmprsk) of the cumulative incidence function and conditional probability function for ICU discharge and in-ICU mortality.(25, 26) P values less than 0.01 were considered significant.(27)

RESULTS

Patient characteristics and outcomes

During the 11-month study period, 1,211 patients were hospitalized in the ICUs (Adult ICU: 863, ER-ICU: 348). Of the 412 included patients, 188 were admitted to the adult ICU and 224 to the ER-ICU. Supplementary Table 3 shows baseline characteristics of included patients in each ICU. There were no significant differences in characteristics between patients in both ICUs, except that in the adult ICU most of the patients had been referred from another ward in the same hospital and the proportion of patients with malignancies was higher. Therefore, we analyzed the data from the ICUs both separately and pooled.

Overall, 192/412 (46.6%) patients had at least one positive culture with *K. pneumoniae,* the remaining 220 patients were free from *K. pneumoniae* on admission and remained so during their ICU stay. One hundred (24.3%) patients already carried *K. pneumoniae* on the day of admission, of whom 78 carried a carbapenem–susceptible *K. pneumoniae* and 22 (5.3%) carried a carbapenem-non-susceptible *K. pneumoniae* strain (Supplementary Figure 1). One hundred patients (32.1%) acquired *K. pneumoniae* during ICU stay, a carbapenem-non-susceptible *K. pneumoniae* strain in 37 cases and a carbapenem-susceptible strain of *K. pneumoniae* by 63 patients. Thus, a total of 59 patients (14.3%) carried a carbapenem-non-susceptible *K. pneumoniae* at a certain moment during their ICU stay. In 44 patients, this *K. pneumoniae* was only found in a screening culture, in five patients only from a clinical specimen, and in ten patients from both screening and clinical samples.

The dynamics of acquisition of *K. pneumoniae* in the ICUs is shown in Figure 1. Patients that acquired a carbapenem-susceptible *K. pneumoniae* had their first positive culture approximately four days sooner than patients that acquired a carbapenem-non-susceptible strain of *K. pneumoniae* (p<0.001). However, the acquisition rate of carbapenem-susceptible *K. pneumoniae* was higher with 41.0 per 1,000 patient-days at risk (adult ICU: 46.1; ER-ICU: 35.9) compared to the acquisition rate of carbapenem-non-susceptible *K. pneumoniae* that was 24.7 per 1,000 patient-days at risk (adult ICU: 22.2; ER-ICU: 27.0).

Patient outcomes were clearly associated with *K. pneumoniae* status. Patients who acquired carbapenem-non-susceptible *K. pneumoniae* during ICU stay had a significantly longer length of stay (median [interquartile range (IQR)]: 11 [8-20] days, adjusted hazard ratio [aHR]: 2.32 [99% confidence interval (CI): 1.35-3.68], p<0.001, Figure 2 and Supplementary Table 4) compared to the other groups of patients, of whom ≥80% were discharged from the ICU within 2-13 days. Interestingly, these latter groups included the patients that were always free from *K. pneumoniae*, and patients that already carried *K. pneumoniae* (either carbapenem-susceptible or carbapenem-non-susceptible) at the time of admission to the ICU and patients who became positive for carbapenem-susceptible *K. pneumoniae* during their stay in ICU (Figure 2).

A longer length of stay was also associated with mechanical ventilation ≥5 days (median [IQR]: 10 [7-15], aHR: 2.79 [CI_{99}]: 1.80-4.34], p<0.001, Supplementary Table 4) and use of a urinary catheter ≥5 days (median [IQR]: 8 [5-12], aHR: 3.88 [CI_{99}: 2.14-7.04], p<0.001, Supplementary Table 4) during ICU stay.

However, the acquisition of *K. pneumoniae* was not associated with in-ICU mortality, 30.5% of patients that remained free of *K. pneumoniae* died versus 17.5% and 43.2% of patients that acquired a carbapenem-susceptible or non-susceptible *K. pneumoniae* strain, respectively, during their ICU stay (Supplementary Table 5, adjusted Odds Ratio [aOR]: 0.40 [99% CI: 0.14-1.13], p=0.023 and 1.03 [0.36-2.97], p=0.937). Interestingly, the group of patients that carried a carbapenem-susceptible strain of *K. pneumoniae*, either on admission or acquired during ICU stay, had the lowest observed mortality rates (24.3% and 17.5%, respectively), even lower than the 30.5% mortality observed among those patients that were always negative for this species, but this difference did not reach statistical significance. However, when compared to patients that had a carbapenem-non-susceptible isolate of *K. pneumoniae*, either on admission or during their ICU stay, the ICU survival of patients with carbapenem-susceptible strains was significantly higher (aHR: 2.57 [99% CI: 1.07-6.17], p=0.005, Figure 3). Importantly, the admission SIRS and qSOFA scores of patients with or without *K. pneumoniae* acquisition did not differ (Table 1), indicating that a difference in the risk of dying was not present at the time of ICU admission but emerged later during their ICU stay (SIRS:

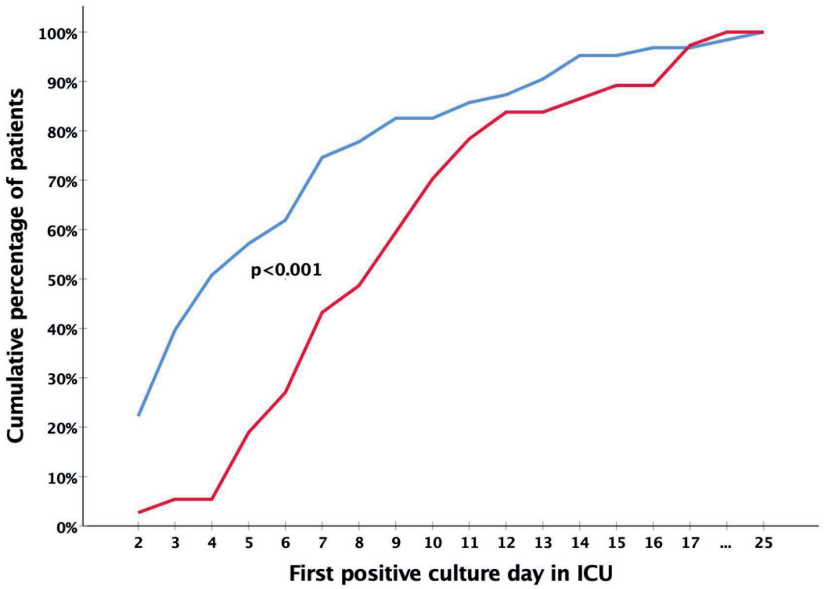

Figure 1. Rate of acquisition of carbapenem-susceptible and -non-susceptible *K. pneumoniae* in ICUs. Acquisition dynamics of carbapenem-susceptible and –non-susceptible *K. pneumoniae* during ICU stay. The blue line represents the cumulative percentage of patients by first day of culture being positive for carbapenem-susceptible *K. pneumoniae* during ICU stay. The red line represents the cumulative percentage of patients by first day of culture being positive for carbapenem-non-susceptible *K. pneumoniae* during ICU stay. P value was calculated using independent samples-Mann Whitney U test. In total, data from 100 patients are included in this figure.

crude Odds Ratio [cOR]: 1.69 [99% CI: 0.55-5.22], p=0.230; qSOFA: cOR: 1.45 [99% CI: 0.68-3.08], p=0.211, Supplementary Table 5).

The competing risk estimates analysis also revealed that the incidence of death was higher in patients with a carbapenem-non-susceptible isolate of *K. pneumoniae* (p=0.006), and the incidence of being discharged alive from ICU was higher for patients with a carbapenem-susceptible *K. pneumoniae* (p=0.0005) (Supplementary Figure 2). Patients that acquired a carbapenem-non-susceptible *K. pneumoniae* during ICU stay were more likely to have had prior exposure to antibiotics, especially carbapenems, and they were more likely to have had a medical indication for their admission to the ICU (Table 1).

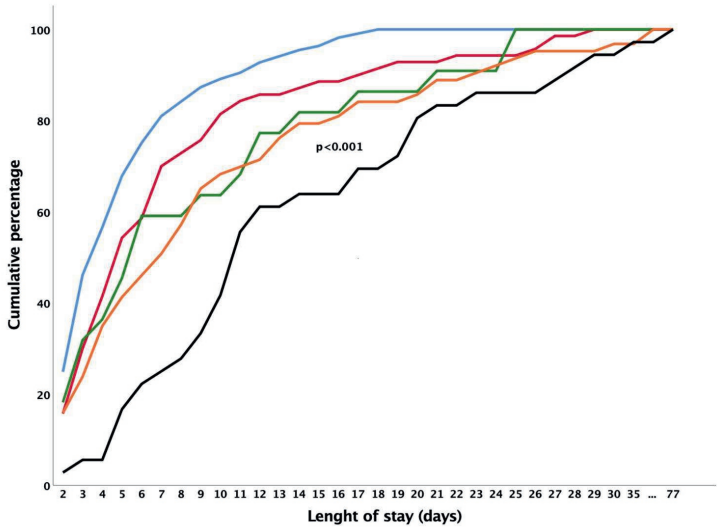

Figure 2. Cumulative percentage of length of stay according to *K. pneumoniae* status. Cumulative length of ICU stay of patients based on their *K. pneumoniae* status. Length of stay (days) represent total days patients were hospitalized in the ICU. The blue line represents patients that were always *K. pneumoniae* negative during their ICU stay. The red line represents patients already positive for carbapenem-susceptible K. pneumoniae on the day of admission. The green line represents patients already positive for carbapenem-non-susceptible *K. pneumoniae* on the day of admission. The orange line represents patients that acquired carbapenem-susceptible *K. pneumoniae* during ICU stay and the black line represents patients that acquired carbapenem-non-susceptible *K. pneumoniae* during ICU stay. The length of stay of patients that became positive with carbapenem-non-susceptible *K. pneumoniae* during ICU stay was longer than that of the other groups (Cox regression, $P < 0.001$).

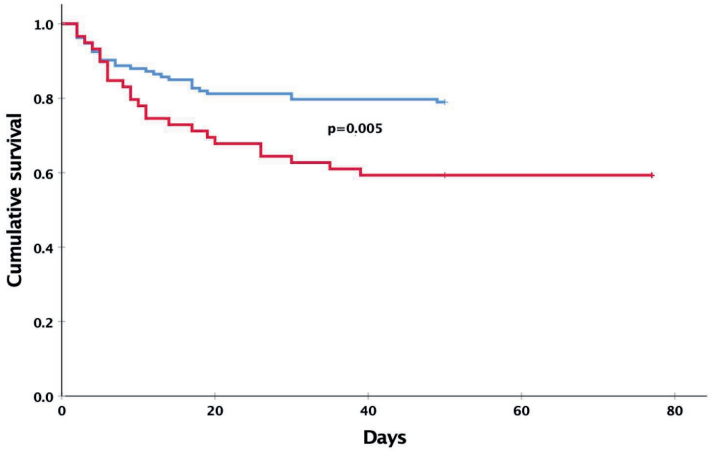

Figure 3. Survival of patients according to their *K. pneumoniae* status. Survival of patients with carbapenem-non-susceptible *K. pneumoniae* (on admission or acquired during ICU stay) (red line) compared with the survival of patients that had carbapenem-susceptible *K. pneumoniae* (on admission or acquired during ICU stay) (blue line) in their screening and/or clinical cultures. P value was calculated using logistic regression.

Table 1. Patient characteristics and outcomes according to their *Klebsiella pneumoniae* status

	Group 1	Group 2	Group 3	Group 4	Group 5	p value
	220	70	22	63	37	
Age (years), median (IQR)	46 (33-58)	49 (33-58)	31.5 (25-49)	50 (38-58)	47 (35.5-62)	0.036
Gender (%)						0.533
Male	108 (49.1)	35 (50)	13 (59.1)	35 (55.6)	23 (62.2)	
Female	112 (50.9)	35 (50)	9 (40.9)	28 (44.4)	14 (37.8)	
Underlying diseases (%)						
Cardiovascular						0.556
Yes	10 (4.5)	64 (91.4)	1 (4.5)	5 (7.9)	3 (8.1)	
No	210 (95.5)	6 (8.6)	21 (95.5)	58 (92.1)	34 (91.9)	
Cerebrovascular						0.026
Yes	11 (5.0)	2 (2.9)	3 (13.6)	8 (12.7)	5 (13.5)	
No	209 (95.0)	68 (97.1)	19 (86.4)	55 (87.3)	32 (86.5)	
Chronic kidney disease						0.068
Yes	17 (7.7)	7 (10.0)	0	1 (1.6)	0	
No	203 (92.3)	63 (70.0)	22 (100)	62 (98.4)	37 (100.0)	
Diabetes mellitus						0.138
Yes	12 (5.5)	10 (14.3)	1 (4.5)	6 (9.5)	4 (10.8)	
No	208 (94.5)	60 (85.7)	21 (95.5)	57 (90.5)	33 (89.2)	
Malignancy						0.717
Yes	68 (30.9)	20 (28.6)	4 (18.2)	16 (25.4)	10 (27.0)	
No	152 (69.1)	50 (71.4)	18 (81.8)	47 (74.6)	27 (73.0)	
Indication for ICU admission (%)						0.005
Medical	64 (29.1)	21 (30.0)	10 (45.5)	23 (36.5)	22 (59.5)	
Surgical	156 (70.9)	49 (70.0)	12 (54.5)	40 (63.5)	15 (40.5)	
Referral from (%)						0.378
Other ward this hospital	115 (52.3)	36 (51.4)	15 (68.2)	33 (52.4)	23 (62.2)	
Other hospital	40 (18.2)	14 (20.0)	2 (9.1)	11 (17.5)	10 (27.0)	
Directly from Emergency Unit	65 (29.5)	20 (28.6)	5 (22.7)	19 (30.2)	4 (10.8)	
Antibiotic exposure (pre-ICU admission)						
Any antibiotic (%)	163 (74.1)	54 (77.1)	18 (81.8)	44 (69.8)	32 (86.5)	0.365
Carbapenem (%)	44 (20.0)	3 (4.3)	7 (31.8)	12 (19.0)	13 (35.1)	<0.001
SIRS Score, (%)						0.598
Score $\geq$2	200 (91.0)	63 (70.0)	22 (100.0)	59 (93.7)	33 (89.2)	
Score <2	20 (9.0)	7 (10.0)	0	4 (6.3)	4 (10.8)	

	Group 1	Group 2	Group 3	Group 4	Group 5	p value
	220	70	22	63	37	
qSOFA Score, (%)						0.971
Score ≥2	179 (81.4)	56 (80.0)	17 (77.3)	51 (81.0)	31 (83.2)	
Score <2	41 (18.6)	14 (20.0)	5 (22.7)	12 (19.0)	6 (16.2)	
Procedures (during ICU admission)						
Mechanical ventilation used (%)	199 (90.5)	59 (84.3)	20 (90.9)	57 (90.5)	36 (97.3)	0.314
Mechanical ventilation (days)						<0.001
≥5 days	74 (33.6)	30 (42.9)	12 (54.5)	34 (54.0)	32 (86.5)	
<5 days	146 (66.4)	40 (57.1)	10 (45.5)	29 (46.0)	5 (13.5)	
Central venous catheter used (%)	193 (87.7)	62 (88.6)	19 (86.4)	53 (84.1)	36 (97.3)	0.343
Central venous catheter (days)						<0.001
≥5 days	96 (43.6)	40 (57.1)	12 (54.5)	42 (66.7)	33 (89.2)	
<5 days	124 (56.4)	30 (42.9)	10 (45.5)	21 (33.3)	4 (10.8)	
Urinary catheter (%)	220 (100.0)	70 (100.0)	22 (100.0)	63 (100.0)	37 (100.0)	NA
Urinary catheter (days) median (IQR)						
≥5 days	112 (50.9)	44 (62.9)	15 (68.2)	46 (73.0)	34 (91.9)	<0.001
<5 days	108 (49.1)	26 (37.1)	7 (31.8)	17 (27.0)	3 (8.1)	
Antibiotic therapy (during ICU admission)						
Any antibiotic (%)	217 (98.2)	70 (100.0)	21 (95.5)	62 (98.4)	36 (100.0)	0.474
Carbapenem (%)	110 (49.8)	26 (37.1)	13 (59.1)	29 (46.0)	21 (58.3)	0.179
Outcomes						
Length of stay (days), median (IQR)	4 (2-6)	5 (3-9)	6 (3-13)	7 (4-13)	11 (8-20)	<0.001
Death (%)	67 (30.3)	17 (24.3)	8 (36.4)	11 (17.5)	16 (44.4)	0.054

Abbreviations: ICU, Intensive Care Unit; IQR, Interquartile range; NS, Non-Susceptible; qSOFA, quick Sepsis-related Organ Failure Assessment; SIRS, Systemic Inflammatory Response Syndrome; S, Susceptible.

Group 1: No *K. pneumoniae* on admission and negative for *K. pneumoniae* during ICU admission.

Group 2: Carbapenem-S *K. pneumoniae* on admission, no carbapenem-NS *K. pneumoniae* acquisition during ICU admission.

Group 3: Carbapenem-NS *K. pneumoniae* on admission, considered as positive during ICU admission* (* regardless of results of follow-up cultures).

Group 4: No *K. pneumoniae* on admission, acquisition of carbapenem-S *K. pneumoniae* during ICU admission.

Group 5: Either no *K. pneumoniae* or carbapenem-S *K. pneumoniae* on admission, acquisition of carbapenem-NS *K. pneumoniae* during ICU admission.

Significance was calculated using One-way ANOVA, Pearson Chi Square and Fisher's Exact Test.

A p-value less than 0.01 was considered statistically significant.

Phenotypic and molecular characterization of carbapenem-non-susceptible K. pneumoniae

Overall, 99/370 (26.8%) isolates from 59/192 (30.7%) patients were found to be non-susceptible to carbapenems. In addition, one (water from suction connector) out of 31 *K. pneumoniae* isolates cultured from the environment (400 samples taken) was carbapenem-non-susceptible. None of 24 *K. pneumoniae* isolates cultured from HCWs (out of 167 screened) were found to be carbapenem-non-susceptible. Thus, a total of 100 carbapenem-non-susceptible isolates was further subjected to phenotypic and molecular analyses. The phenotypic detection test indicated that 96/100 (96%) isolates produced a MBL. PCRs of carbapenemase genes demonstrated the presence of the bla_{NDM} gene in these 96 carbapenem-non-susceptible isolates, including isolates from patients and the one from the environment. None of the 100 isolates was positive for either the bla_{KPC} or $bla_{\text{OXA-48}}$ gene. Four carbapenem-non-susceptible strains apparently contained another resistance mechanism, which was not further investigated, they remained relatively susceptible to carbapenems (MIC meropenem 2-4 mg/L). The string test was positive for only four isolates from three patients, one of whom deceased in ICU.

Clonal relatedness

Raman spectroscopy analysis performed for 100 isolates revealed the presence of multiple types within this collection of *K. pneumoniae.* In total, 48 Raman types were identified. There were three major clusters (Supplementary Figure 3), the largest cluster (CIPTOKPN24) consisted of 20 isolates obtained from 13 patients (screening and clinical specimens). Strains belonging to the dominant cluster CIPTOKPN24 were present in both ICUs throughout the study period, whereas other clones seemed to wax and wane with time (Figure 4).

A total of 97 clinical (two isolates were lost during storage) and 1 environmental isolate were further analyzed using MLVA genotyping, identifying 30 different genotypes (Figure 5). The most dominant clone accounted for 26.5% (n=26) of all isolates, whereas 19 isolates (19.4%) were of a unique genotype, the remaining 53 isolates belonged to 20 other genotypes. Clustering of strains by Raman spectroscopy into three dominant groups was concordant with clustering by MLVA, e.g. the 20 Raman CIPTOKPN 24 strains all belonged to a single MLVA clonal complex. Likewise, the four Raman CIPTOKPN 30 strains belonged to a single MLVA clonal complex as did 8/10 CIPTOKPN 27 isolates.

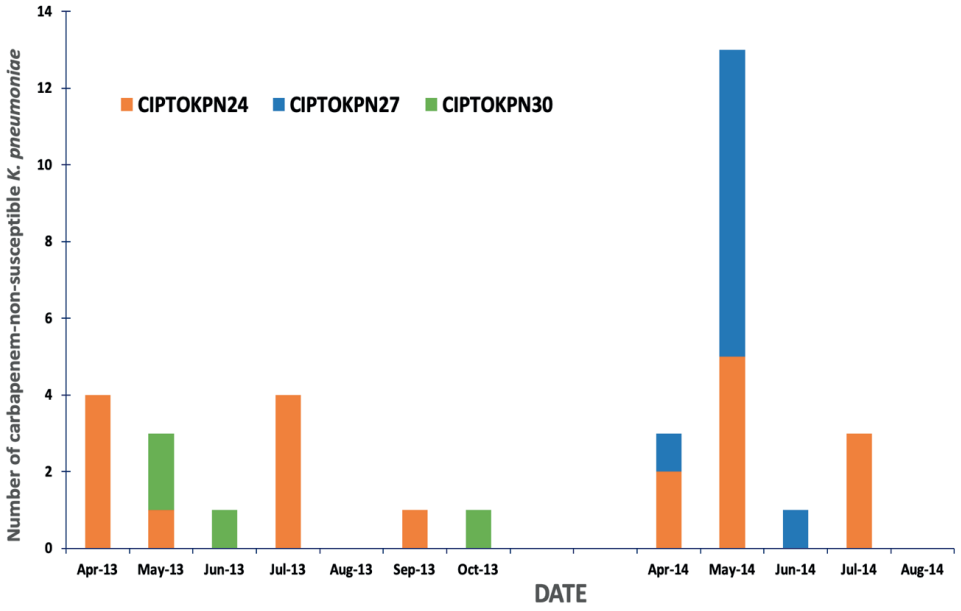

Figure 4. Persistence of prevalent clones of carbapenem-non-susceptible *K. pneumoniae* in ICUs, as determined by Raman spectroscopy typing. Endemicity of the three largest clusters, as determined by Raman spectroscopy, of carbapenem-non-susceptible *K. pneumoniae* in ICUs, April–October 2013 and April–August 2014. The orange bars represent cluster CIPTOKPN24. The blue bars represent CIPTOKPN27. The green bars represent CIPTOKPN30. The x-axis indicates time periods of the study (by week, April 2013-Oktober 2013 and April 2014–August 2014). The y-axis indicates number of isolates.

DISCUSSION

This is the first report of a study on the clinical and molecular epidemiology of carbapenemase-producing *K. pneumoniae* in ICUs in Indonesia. These two ICUs can be considered to have endemic carbapenem-non-susceptible *K. pneumoniae* whose acquisition by patients is associated with prolonged ICU stay and, possibly, an increased risk of dying.

The dissemination of *K. pneumoniae* isolates harboring carbapenemase genes, continues unabated, and reports describing these isolates are emerging from different parts of the world, including Southeast Asia. (1, 4, 28). Colonization and infection with carbapenem-resistant *K. pneumoniae* has been reported in Singapore (29). In Malaysia, the National Surveillance of Antimicrobial resistance found carbapenem resistance rates among *K. pneumoniae* to increase from 0.5% (11,935 isolates tested) in 2010 to 1.6% (27,911 isolates tested) in 2014 (30). The Philippines Department of Health's Research Institute reported a rate of 11.9% in 2015 (30). Morocco (31), Italy (32), and India have likewise shown dramatic increases over time (30). Similar to these studies, we found that 59/412 (14.3%) of the patients in the ICU carried a

carbapenem-non-susceptible *K. pneumoniae*. By screening on ICU admission 5.3% of patients were already colonized with carbapenem-non-susceptible *K. pneumoniae* prior to their admission to the ICU.

This suggests that patients may become colonized with such strains elsewhere in the same hospital or in another hospital from which they are referred, or may come with such strain directly from the community, possibly having acquired their strain during a previous healthcare contact or indirectly from exposure to reservoirs or relatives carrying such strains (33)

Screening cultures can, therefore, be considered helpful for early detection and infection control. It may also be useful to guide rational antibiotic use, since previous studies have shown that colonization with a carbapenem-resistant *K. pneumoniae* is a risk factor for subsequent infection. (34) (35) However, during our study, carbapenem-non-susceptible *K. pneumoniae* strains were not isolated from blood cultures (data not shown).

Our data also show that patients may acquire carbapenem-non-susceptible *K. pneumoniae* during ICU stay in the setting of our study and that these acquisitions are associated with significantly longer ICU stay. At the level of significance chosen, the acquisition of *K. pneumoniae* strains, whether carbapenem susceptible or not, was not associated with mortality when compared to patients who remained free of *K. pneumoniae*. In contrast, the study from Dautzenberg et al. (2015) showed patients colonized with carbapenemase-producing Enterobacteriaceae to have on average a 1.79 times higher hazard of dying in ICU than non-colonized patients, primarily because of an increased length of stay (36). A study in Singapore reported that cases with carbapenem-resistant strains of Enterobacteriaceae had ~3.5 times increased odds of fatality adjusted for length of hospital stay. (29)

Interestingly, our study shows that the risk of dying among ICU patients who were culture-positive for carbapenem-non-susceptible *K. pneumoniae* on admission or during ICU stay was significantly, 2.57 times, higher than among patients who were culture-positive for a carbapenem-susceptible *K. pneumoniae*. The observed ICU fatality rates were indeed highest (44.4%) among those acquiring carbapenem-non-susceptible *K. pneumoniae* and lowest (17.5%) amongst the patients acquiring carbapenem-susceptible strains of *K. pneumoniae* during their ICU stay. Since risk of mortality during ICU stay is influenced by many factors, as reported by other international findings (32, 37, 38), we cannot readily explain why susceptible *K. pneumoniae* acquisitions seem to be a proxy for protection whereas non-susceptible *K. pneumoniae* acquisitions may be predictive of a fatal outcome. Probably, exposure to carbapenem antibiotics, and the underlying reasons for this, may be important determinants in this respect.

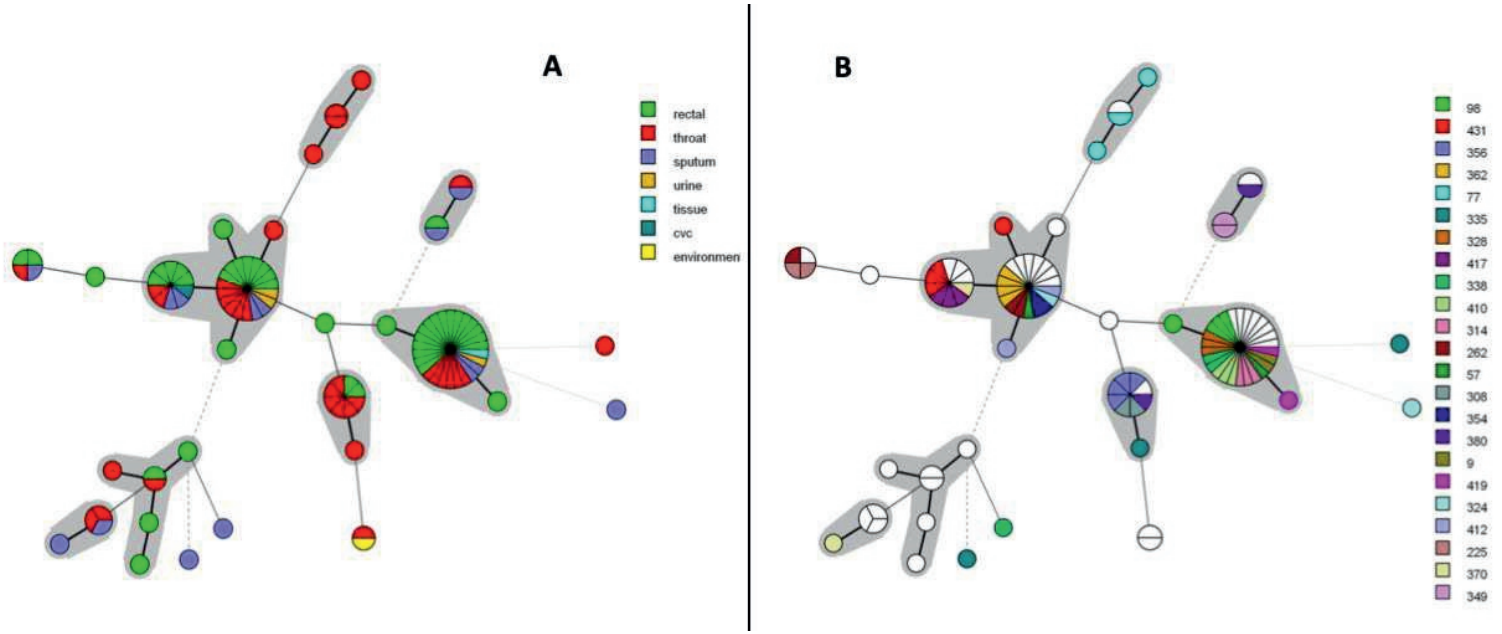

Figure 5. MLVA minimum spanning trees of carbapenem-non-susceptible *Klebsiella pneumoniae*. Minimum spanning tree analysis of *K. pneumoniae* isolates based on clustering at the VNTR loci. Clusters of genotypes differing in only one marker are indicated with a grey background. Panel a: Colours correspond to specimens from which *K. pneumoniae* isolates were cultured. Panel b: Distribution of genotypes per patient. Each colour, except white, indicates a different patient. Only patients with 2 or more isolates are presented in this manner. Patients that had only one isolate of a carbapenem-non-susceptible *K. pneumoniae* are indicated by the colour white.

The *bla*$_{NDM}$ gene was the most prevalent carbapenemase gene as it was detected in 96 isolates, including one from the environment. Carbapenem resistance due to *bla*$_{KPC}$-like and *bla*$_{OXA-48}$-like genes was not detected. In South Asia (India and Pakistan) the NDM-1 gene was initially found and currently, this enzyme is by far the most prevalent and widely distributed carbapenem degrading enzyme in the world, including in Southeast Asia. (1, 3-6, 8-10, 18, 28-31)

Walsh et al. (2011) have also found the presence of NDM-1 β-lactamase-producing bacteria, including *K. pneumoniae*, from waste seepages samples in Indian community. (33) Taking our study and the recent report on NDM-1 in carbapenem-non-susceptible Enterobacteriaceae from urinay tract samples of hospitalized patients in Surabaya, Indonesia, into account, we suspect that this carbapenemase gene is widespread in hospitals in Indonesia.(39)

Carbapenem-susceptible and -non-susceptible *K. pneumoniae* that colonize or infect ICU patients may originate from the patient her/himself, but may also come from contaminated hospital equipment and environment, staff and other patients. In this study *K. pneumoniae* was found in the ICU environment, including one endemic strain that was carbapenem-non-susceptible. Predictably, *K. pneumoniae* was also cultured from throat and rectal swabs of ICU personnel, although none of those isolates was carbapenem-non-susceptible. However, we cannot exclude personnel as a source or vector of *K. pneumoniae* since personnel was only screened once during this study and other body parts (e.g. hands) or clothes were not sampled, limiting the sensitivity of this part of the survey. A recent study in China found that almost 9% of medical personnel in ICU carried multidrug-resistant Gram-negative bacteria on their hands.(40) Transmission of the bacteria may occur with many risk factors involved. (1, 3, 41) Multiple studies reported outbreaks of carbapenem-resistant *K. pneumoniae* that were associated with environmental contamination.(42-44) We performed Raman spectroscopy and MLVA to assess clonal relatedness. These analyses revealed three major clusters by Raman typing, with the largest one (CIPTOKPN24), persisting in both ICUs throughout the whole study period. However, many carbapenem-non-susceptible strains of *K. pneumoniae* cultured in this study were of a unique Raman type or belonged to small clusters that waxed and waned quickly, indicating both endemicity of certain clones in the ICUs but also regular new introductions and rapid loss of many clones over time. This epidemiologic information can and should be applied when designing interventions to reduce the acquisition of carbapenen-resistant *K. pneumoniae* in ICUs in Indonesia and in similar settings elsewhere.

Although not used routinely, Raman spectroscopy typing can be valuable for discriminating types of strains within a species (21,22,23). Here we showed typing by Raman spectroscopy to yield *K. pneumoniae* strain clustering compatible with clustering based on MLVA genotyping. However, Raman typing results at a given site cannot be directly compared with

results generated or published elsewhere, and are, thus, not easily shared or pooled. Considering the modification that we made to MLVA marker VNTR58, the genotype of the most dominant clone in our study was of genotype "4-2.4-3-4-4.3-1-12-19". This genotype would translate into "4-3-3-4-5-1-12-19" based on the original MLVA typing method for *K. pneumoniae* [24], but this genotype was not observed by Brink et al.(24). On the other hand, the second and third most dominant genotype "5-3-3-4-6-1-9-12" and "5-3-3-5-6-1-9-12" match genotype "5-3-3-4-6-1-9-1" and a single locus variant thereof from Brink et al. that involves isolates with MLST sequence type ST147. *K. pneumoniae* ST147 strains belong to a relatively common NDM-positive *K. pneumoniae* lineage and have been found in multiple countries across several continents, almost all of which were isolated from humans. (45, 46) Unlike in the original paper, in our approach the non-integer alleles were considered as separate alleles. As a result, the total number of alleles per marker will increase for several markers and as a result this may benefit the overall discriminatory power of the MLVA method. Indeed, in our study (exceeding the ones reported here), we observed several different genotypes that would have been assigned identical when the non-integer alleles would have been ignored by 'rounding off' their values using adjusted and broadened binsets (results not shown).

There were some limitations in this study. First, as no colometric agar plate was used for the screening cultures, overgrowth of carbapenem-susceptible but cefotaxime-resistant isolates could have led to overlooking CNKP. However, all morphologically different colonies were checked, and this was done by trained and experienced microbiology technicians. Colometric media are expensive, therefore, could not be used in this study. Also, cefotaxime-susceptible OXA-48-producing isolates could have been missed with our screening method. Nevertheless, based on the isolates found in the clinical cultures, it is unlikely that these were playing a major role in the epidemiology. Of note, the OXA-48 PCR was not able to detect OXA-54 and OXA-436, but given the results of the phenotypic detection method, and epidemiology in countries nearby, these are also not suspected. Second, only two ICUs in one tertiary care academic hospital participated, which does not permit results to be called representative for all ICUs in Indonesia. Third, the study was performed more than 5 years ago, hence the epidemiology of CNKP in Indonesia may be different now. Finally, the limited rate of sampling of the environment and of personnel (as opposed to patients) may have undervalued their role in the chain of transmission and acquisition of carbapenem-non-susceptible *K. pneumoniae.*

CONCLUSIONS

In summary, this study is the largest to date that describes the characteristics and epidemiology of, and outcome associated with carbapenem-non-susceptible *K. pneumoniae* in ICUs in Indonesia. Colonization or infection with carbapenem-non-susceptible *K. pneumoniae*

during hospitalization was independently associated with prolonged LOS in the ICU, and may affect survival during ICU stay. Prevention of colonization by and infection from these multidrug-resistant strains requires interventions directed to source control and limiting the introduction and transmission of such strains to and between patients.

DECLARATIONS

Ethics and Regulatory Considerations

The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the research on 17th September 2012, No: 561/PT02.FK/ETIK/2012, No: 757/UN2.F1/ETIK/X/2014.
A Material Transfer Agreement (MTA) was reviewed and approved by the Director of National Institute Research and Development, Ministry of Health (No: LB.02.01/I.9.4/8500/2013).
Trial registration: The study was registered at Netherlands Trial Register http://www.trialregister.nl (No: 5541). Candidate number: 23527, NTR number: NTR5541, retrospectively registered: NTR: 22 December 2015

Consent for publication

Informed consent was documented by the use of a written consent form approved by the Ethics Committee Faculty of Medicine Universitas Indonesia / Dr. Cipto Mangunkusumo General Hospital and signed and dated by the subjects/guardians and by the person who conducted the informed consent discussion and two witnesses. The signature confirmed the consent was based on information that had been understood.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

YRS is an awardee of the DIKTI-NESO Scholarship by The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, and Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands.
All authors report no conflict of interest relevant to this article.

Funding

This work was supported by 'The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia' and 'Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands'.

Authors' contributions

YRS, AK, HAV, and JAS conceived the study and participated in design of the study. YRS, RS, and DA participated in acquisition of data.

YRS, WHFG, CHWK, HAV, and JAS performed data analysis and interpreted the data.

YRS, CHWK, HAV, and JAS drafted the article.

All authors participated in critically revising the draft.

All authors read and approved the final manuscript.

Acknowledgements

We are thankful to The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, Dean of Faculty of Medicine Universitas Indonesia, Board of Directors of Dr. Cipto Mangunkusumo National General Hospital Jakarta Indonesia, Department of Medical Microbiology and Infectious Diseases, especially Michelle de Regt and Liesbeth van der Zwaan for their support in the laboratory, Erasmus MC in Rotterdam, The Netherlands and Critical Care Division, Department of Anesthesia and Intensive Care, Faculty of Medicine Universitas Indonesia / Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia.

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SUPPLEMENTAL MATERIALS

Supplementary Table 1. List of environmental samples

Sample site	Number of samples	
	Adult ICU	ER-ICU
Wash basin on ICUs ward	10	5
Monitor	14	10
Ventilator	15	5
Ambu bag	8	
Stethoscope	10	9
Drawer handle bedside cabinet	21	
Plastic multi-purpose container next to each bed	18	8
Stainless steel container	8	15
Flowmeter	15	
Infusion stand	11	8
Infusion pump	9	6
Bed rails	20	14
Tap water (wash basin on ICU ward)	10	8
Chart paper on bedside cabinet	11	6
Bedside cabinet table	15	11
Cleaning room: wash basin	9	
Cleaning room: sink countertop	3	
Cleaning room: mug	2	
Cleaning room: dish rack	3	
Mattress	5	4
Comb	3	
Water from siphon of wash basin	10	5
Water from mug next to each bed	10	6
Massage oil	5	
Chlorine solution after use	3	
Cleaning wipes	3	4
Wall	3	2
Drawer of bedside cabinet	3	
Water from suction	7	
Suction connector/container	3	
Water from humidifier	1	
Water after cleaning a floor	2	
Floor	2	
Nurse station	1	1

Abbreviations: ER-ICU. Emergency room Intensive Care Unit; ICU, Intensive Care Unit

Supplementary Table 2. Amplification primers used for MLVA typing. FAM, VIC, NED and PET represent the different fluorescent labels used for detection of the markers.

Marker	Forward primer (5'-3')	Reverse primer (5'-3')	Conc (µM)	Reference
VNTR52	FAM-TTTGGCGGCAGCGGTTTCCC	GCCAGAAAAAGGCGCGCAGC	0.4	(1)
VNTR45	FAM-CGCTGACACATTGACGAAAACAGAGA	ATGAATATTGCCCAGTTTCTGGAACAA	0.4	(1)
VNTR53	FAM-CGCAGAAGAAAGCGGAAG	TGTTTTAGGCGCATTCTTACC	0.4	(1)
VNTR51	VIC-CCGCCGCGCCATCGTTAGAT	TCAACGCGCCCAGCTGAACC	0.25	(1)
VNTR60	VIC-CGGTACGAATCTGTTGGATTAAG	GGCCTTCTTCCGGGTCTAT	0.2	(1)
VNTR10	VIC-AGCGCGCAGACGATGAGCAG	AGCCCCGCAGTGGGGTTACT	0.4	(1)
VNTR27	NED-CAGCGTCAGCGCCAGACCAA	CCATGGCCGGCCTGTGGTTT	0.4	(1)
VNTR58	CGGAAGACGTGGTTGATATG	PET-GCCGAACATATCCTTGATCC		This study

Modification of the original MLVA typing method of Brink et al. [1]

In our hands, MLVA marker VNTR58 yielded sizing results that were difficult to interpret as the cumulative result of DNA sequence variations within the amplified product as well as due to size variation of a poly-T stretch in the flanking region of the repeat. To eliminate part of these variations we developed alternative primers (Supplementary Table 2, below) that amplified a much smaller product with sizing results that were more easily interpreted. Since this approach with new primers invalidated the reference values from Brink et al. for correlating the PCR amplicon sizes to the numbers of repeats in this marker, representative alleles were sequenced by Sanger sequencing to determine the number of repeats [1]

In our collection of isolates, we observed multiple alleles that deviated in size from the expected values as the result of insertions/deletions in the flanking region of the repeat region or in the repeat region itself. Specifically, marker VNTR10 involves a 57 bp repeat region but we also observed amplification products that were 12 bp shorter than the expected sizes. DNA sequence analysis confirmed that this was the result of a 12 bp deletion in the flanking region of the repeat (results not shown). Instead of adjusting the binset for such alleles as described in the original Klebsiella pneumoniae MLVA paper [1], we assigned these according to the recommendations for assigning non-integer alleles in human forensics [2].

Consequently, allele n.45 for this marker represents a PCR product size that corresponds to n repeats + 45 additional base pairs. Similarly, in marker VNTR60 (amplifying a 7 bp repeat) n.3 alleles were observed, in marker VNTR45 (amplifying a 12 bp repeat) n.4 alleles were observed and in marker VNTR58 n.1 alleles were observed.

References

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Supplementary Table 3. Baseline characteristics of 412 patients admitted to the adult or emergency Room (ER) ICUs , and enrolled in this study.

	Adult ICU	ER-ICU	p value
Number of patients enrolled	188	224	
Age (years). median (IQR)	49 (38-58)	43 (30-58)	0.041
Gender			
Male (%)	91 (48.4)	123 (54.9)	0.188
Female (%)	97 (51.6)	101 (45.1)	
Underlying diseases	126 (54.8)	104 (45.2)	0.000**
Cardiovascular (%)	12 (6.4)	13 (5.8)	0.806
Cerebrovascular (%)	10 (5.3)	19 (8.5)	0.211
Chronic kidney disease (%)	9 (4.8)	16 (17.1)	0.319
Diabetes mellitus (%)	15 (8)	18 (8)	0.983
Malignancy (%)	80 (42.6)	38 (17)	0.000**
Indication for ICU admission			0.039
Medical (%)	54 (28.7)	86 (38.4)	
Surgical (%)	134 (71.3)	138 (61.6)	
Referral from			0.000**
Other ward this hospital (%)	144 (76.6)	78 (34.8)	
Other hospital (%)	20 (10.6)	57 (25.4)	
Directly from Emergency Unit (%)	24 (12.8)	89 (39.8)	
Antibiotic exposure (before admission to ICU)			
Any antibiotic (%)	146 (77.7)	165 (73.7)	0.349
Carbapenem (%)	40 (21.3)	39 (17.4)	0.321
SIRS Score. (%)			0.992
Score ≥2	172 (91.5)	205 (91.5)	
Score <2	16 (8.5)	19 (8.5)	
qSOFA Score. (%)			0.158
Score ≥2	158 (84.0)	176 (78.6)	
Score <2	30 (16.0)	48 (21.4)	
Procedures (during ICU admission)			
Mechanical ventilation (%)	170 (90.4)	201 (89.7)	0.815
Mechanical ventilation (days), median (IQR)	4 (1.5-9)	3 (2-7)	0.591
≥5 days (%)	87 (26.3)	95 (42.4)	0.431
<5 days (%)	101 (53.7)	129 (57.6)	
Central venous catheter (%)	166 (88.3)	197 (87.9)	0.913
Central venous catheter (days). median (IQR)	5.5 (3-10)	5 (3-8.5)	0.150
≥5 days (%)	106 (56.4)	117 (52.2)	0.400
<5 days (%)	82 (43.6)	107 (47.8)	
Urinary catheter (%)	188 (100)	224 (100)	N/A
Urinary catheter (days), median (IQR)	6 (3-11)	5 (3-9)	0.181
≥5 days (%)	118 (62.8)	133 (59.4)	0.486

	Adult ICU	ER-ICU	p value
<5 days (%)	70 (37.2)	91 (40.6)	
Antibiotic therapy (during ICU admission)			
Any antibiotic (%)	188 (100)	218 (97.3)	0.034
Carbapenem (%)	99 (52.7)	100 (44.6)	0.105
Outcomes			
Length of stay (days). median (IQR)	5 (3-10.75)	5 (3-8)	0.024
Death (%)	52 (27.7)	67 (29.9)	0.616

Abbreviations: ER-ICU. Emergency room Intensive Care Unit; ICU, Intensive Care Unit; IQR, Interquartile range; qSOFA, quick Sepsis-related Organ Failure Assessment; SIRS, Systemic Inflammatory Response Syndrome.

**p<0.01

Supplementary Table 4. Variables associated with length of stay among patients with and without carbapenem-non-susceptible *Klebsiella pneumoniae*

	Length of stay Median (IQR)	Univariate analysis				Multivariate analysis			
		P	cHR	99% CI		p	aHR	99% CI	
				Lower	Upper			Lower	Upper
Group (see note below)									
Group 1	4 (2-6)	<0.001	1,00				1.00		
Group 2	5 (3-9)	0.002	1.55	1.08	2.23	0.014	1.41	0.98	2.04
Group 3	6 (3-12)	0.007	1.83	1.02	3.29	<0.001	3.57	1.83	6.94
Group 4	7 (4-13)	<0.001	2.14	1.45	3.15	0.001	1.64	1.10	2.43
Group 5	11 (8-20)	<0.001	3.18	1.96	5.15	<0.001	2.32	1.35	3.68
Gender									
Male	5 (3-10)	0.426	0.92	0.72	1.19				
Female	4 (3-9)		1.00						
Underlying diseases									
Cardiovascular									
Yes	8 (5-12)	0.199	1.31	0.77	2.22				
No	5 (3-9)		1.00						
Cerebrovascular									
Yes	8 (3-14)	0.026	1.54	0.93	2.55	0.851	1.04	0.62	1.77
No	5 (3-9)		1.00				1.00		
Chronic kidney diseases									
Yes	4 (3-6)	0.041	0.65	0.38	1.12	0.605	0.89	0.50	1.58
No	5 (3-10)		1.00				1.00		
Diabetes mellitus									
Yes	7 (4-14)	0.108	1.34	0.84	2.14				
No	5 (3-9)		1.00						

	Length of stay Median (IQR)	Univariate analysis				Multivariate analysis			
		P	cHR	99% CI		p	aHR	99% CI	
				Lower	Upper			Lower	Upper
Malignancy									
Yes	4 (3-9)	0.214	0.87	0.66	1.16				
No	5 (3-9)		1.00						
Indication for ICU admission									
Medical	6 (3-12)	<0.001	1.52	1.15	2.01	0.893	0.98	0.72	1.35
Surgical	4 (3-8)		1.00				1.00		
Referral from									
Other ward this hospital	5 (3-10)	0.197	1.21	0.83	1.77				
Other hospital	5 (3-9)	0.151	1.18	0.88	1.59				
Directly from Emergency Unit	5 (3-8)		1.00						
Antibiotic exposure (before admission to ICU)									
Any antibiotic	5 (3-10)	0.014	1.33	0.99	1.79	0.097	1.22	0.90	1.65
No any antibiotic	4 (3-7)		1.00				1.00		
Carbapenem	8 (3-13)	0.012	1.37	0.99	1.90	0.366	1.14	0.79	1.63
No carbapenem	5 (3-8)		1.00				1.00		
SIRS score									
Score ≥2	5 (3-9)	0.651	1.08	0.69	1.71				
Score <2	4 (2-7)		1.00						
qSOFA									
Score ≥2	5 (3-10)	<0.001	1.62	1.17	2.25	0.031	1.33	0.95	1.88
Score <2	3 (2-6)		1.00				1.00		
Procedures (during ICU admission)									
Mechanical ventilation used	5 (3-10)	<0.001	2.50	1.61	3.89	0.369	1.19	0.73	1.93
No mechanical ventilation used	3 (2-4)		1.00				1.00		

	Length of stay Median (IQR)	Univariate analysis				Multivariate analysis			
		P	cHR	99% CI		p	aHR	99% CI	
				Lower	Upper			Lower	Upper
Mechanical ventilation (days)									
≥5 days	10 (7-15)	<0.001	6.33	4.59	8.73	<0.001	2.79	1.80	4.34
<5 days	3 (2-4)		1.00				1.00		
Central venous catheter used	5 (3-10)	<0.001	2.12	1.42	3.17	0.492	0.88	0.53	1.45
No central venous catheter used	3 (2-5)		1.00				1.00		
Central venous catheter (days)									
≥5 days	9 (6-13)	<0.001	6.30	4.62	8.59	0.015	1.69	0.97	2.93
<5 days	3 (2-3)		1.00				1.00		
Urinary catheter used	5 (3-9)	N/A							
No urinary catheter used	N/A								
Urinary catheter (days)									
≥5 days	8 (5-12)	<0.001	9.87	6.84	14.24	<0.001	3.88	2.14	7.04
<5 days	3 (2-3)		1.00				1.00		
Antibiotic therapy (during ICU admission)									
Any antibiotic (%)	5 (3-9)	0.005	3.20	1.10	9.33	0.302	1.55	0.52	4.62
No any antibiotic	2 (2-3)		1.00				1.00		
Carbapenem (%)	7 (4-12)	<0.001	1.78	1.37	2.32	0.566	1.07	0.78	1.48
No Carbapenem	4 (2-7)						1.00		
Age, correlation coefficient (r)	0.081	0.154	1.00	0.99	1.00	0.655	1.00	0.99	1.01
Mortality	6 (3-12)	0.057	1.23	0.93	1.63	0.226	0.87	0.65	1.17

Abbreviation: aHR, adjusted Hazard Ratio; cHR, crude Hazard Ratio; CI, Confidence Interval; ICU, Intensive Care Unit; IQR, Interquartile Range; LOS, Length of Stay; NS, Non-susceptible; S, Susceptible; SIRS, Systemic Inflammatory Response Syndrome; qSOFA, quick Sepsis-related Organ Failure Assessment .

Group 1: No *K. pneumoniae* on admission and negative for *K. pneumoniae* during ICU admission.

Group 2: Carbapenem-S *K. pneumoniae* on admission, no carbapenem-NS *K. pneumoniae* acquisition during ICU admission.

Group 3: Carbapenem-NS *K. pneumoniae* on admission, considered as positive during ICU admission* (* regardless of results of follow-up cultures).

Group 4: No *K. pneumoniae* on admission, acquisition of carbapenem-S *K. pneumoniae* during ICU admission.

Group 5: Either no *K. pneumoniae* or carbapenem-S *K. pneumoniae* on admission, acquisition of carbapenem-NS *K. pneumoniae* during ICU admission.

A p-value less than 0.01 was considered statistically significant.

Supplementary Table 5. Variables associated with mortality among patients with and without carbapenem-non-susceptible *Klebsiella pneumoniae*

	Univariate analysis						Multivariate analysis			
	Mortality		p	99% CI			p	99% CI		
	N	%		cOR	Lower	Upper		aOR	Lower	Upper
Group (see Note below)										
Group 1	67	30.5		1.00						
Group 2	17	24.3	0.323	0.73	0.33	1.65	0.413	0.76	0.31	1.82
Group 3	8	36.4	0.569	1.31	0.39	4.34	0.757	1.17	0.31	4.43
Group 4	11	17.5	0.045	0.48	0.19	1.23	0.023	0.40	0.14	1.13
Group 5	16	43.2	0.127	1.74	0.68	4.43	0.937	1.03	0.36	2.97
Gender										
Male	66	55.5	0.362	1.22	0.70	2.14				
Female	53	44.5		1.00						
Underlying diseases										
Cardiovascular										
Yes	9	7.6	0.418	1.42	0.47	4.31				
No	110	92.4		1.00						
Cerebrovascular										
Yes	13	10.9	0.049	2.12	0.78	5.81	0.515	1.35	0.42	4.35
No	106	89.1		1.00				1.00		
Chronic kidney diseases										
Yes	13	10.9	0.011	2.87	0.98	8.39	0.043	2.60	0.77	8.78
No	106	89.1		1.00						
Diabetes mellitus										
Yes	11	9.2	0.556	1.25	0.46	3.40				
No	108	90.8		1.00						

	Univariate analysis						Multivariate analysis			
	Mortality		p	99% CI			p	99% CI		
	N	%		cOR	Lower	Upper		aOR	Lower	Upper
Malignancy										
Yes	30	25.2	0.326	0.79	0.42	1.48				
No	89	74.8		1.00						
Indication for ICU admission										
Medical	61	51.3	<0.001	2.85	1.59	5.10	0.016	1.81	0.96	3.40
Surgical	58	48.7		1.00				1.00		
Referral from										
Other ward this hospital	58	48.7	0.798	0.94	0.48	1.83	0.187	0.67	0.31	1.46
Other hospital	30	25.2	0.096	1.69	0.75	3.80	0.891	1.05	0.41	2.68
Directly from Emergency Unit	31	26.1		1.00				1.00		
Antibiotic exposure (before admission to ICU)										
Any antibiotic	100	84.0	0.011	2.05	0.99	4.23	0.320	1.39	0.59	3.24
No any antibiotic	19	16.0		1.00				1.00		
Carbapenem	36	30.3	<0.001	2.52	1.29	4.91	0.116	1.65	0.73	3.76
No carbapenem	83	69.7		1.00				1.00		
SIRS score										
Score ≥2	112	94.1	0.230	1.69	0.55	5.22				
Score <2	7	5.9		1.00						
qSOFA										
Score ≥2	101	84.9	0.211	1.45	0.68	3.08				
Score <2	18	15.1		1.00						
Procedures (during ICU admission)										
Mechanical ventilation used (%)	119	100	N/A							
No mechanical ventilation used (%)	0	0								

	Univariate analysis						Multivariate analysis			
	Mortality		p	99% CI			p	99% CI		
	N	%		cOR	Lower	Upper		aOR	Lower	Upper
Mechanical ventilation (days)										
≥5 days	74	62.2	<0.001	2.82	1.58	5.02	0.001	5.08	1.46	17.7
<5 days	45	37.8		1.00				1.00		
Central venous catheter used (%)	116	97.5	<0.001	7.20	1.51	34.34	0.006	6.38	1.13	36.09
No central venous catheter used (%)	3	2.5		1.00				1.00		
Central venous catheter (days)										
≥5 days	77	64.7	0.006	1.85	1.04	3.29	0.011	0.28	0.08	1.02
<5 days	42	35.3		1.00				1.00		
Urinary catheter used (%)	119	100	N/A							
No urinary catheter used (%)	0	0								
Urinary catheter (days)										
≥5 days	85	71.4	0.005	1.91	1.05	3.50	0.601	1.34	0.32	5.72
<5 days	34	28.6		1.00				1.00		
Antibiotic therapy (during ICU admission)										
Any antibiotic (%)	119	100	N/A							
No any antibiotic	0	0								
Carbapenem (%)	81	68.1	<0.001	3.16	1.75	5.72	0.005	2.06	1.07	3.97
No Carbapenem	38	31.9		1.00				1.00		
Age, median (IQR) (years old)	50	(38-60)	0.009	1.02	1.00	1.04	0.138	1.01	0.99	1.03
LOS, median (IQR) (days)	6	(3-12)	0.058	1.03	0.99	1.06	0.367	0.98	0.94	1.03

Abbreviation: aOR, adjusted Odds Ratio; cOR, crude Odds Ratio; CI, Confidence Interval; ICU: Intensive Care Unit; IQR, Interquartile range; LOS, Length of Stay; NS, Non-susceptible; S, Susceptible; SIRS, Systemic Inflammatory Response Syndrome; qSOFA, quick Sepsis-related Organ Failure Assessment.

Group 1: No *K. pneumoniae* on admission and negative for *K. pneumoniae* during ICU admission.

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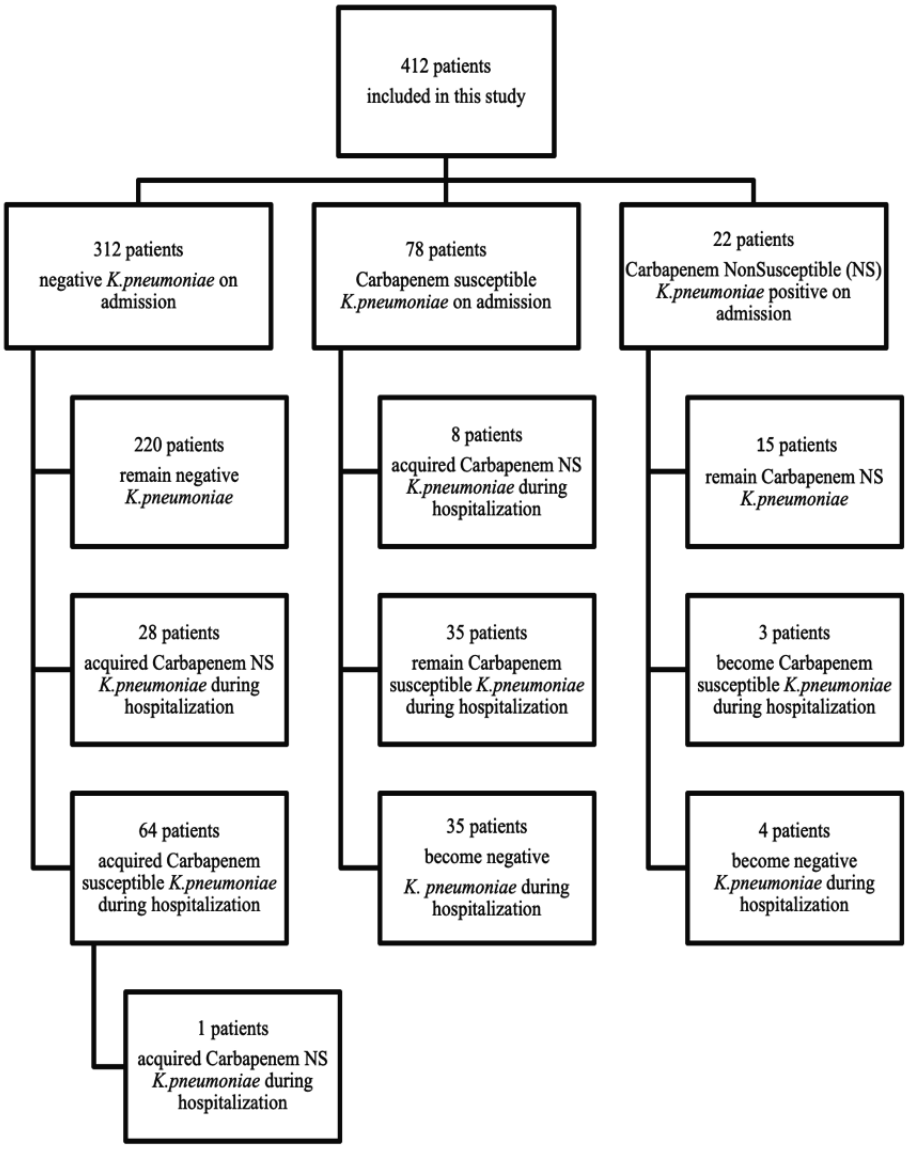
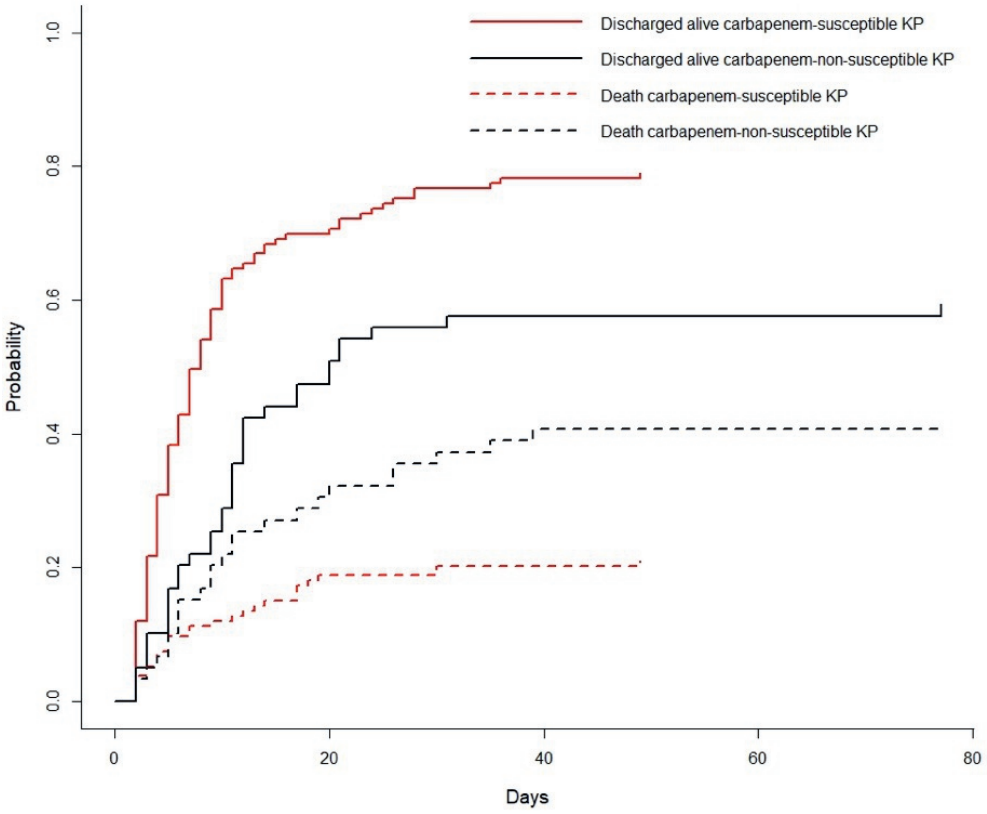
Group 5: Either no *K. pneumoniae* or carbapenem-S *K. pneumoniae* on admission, acquisition of carbapenem-NS *K. pneumoniae* during ICU admission.

A p-value less than 0.01 was considered statistically significant.



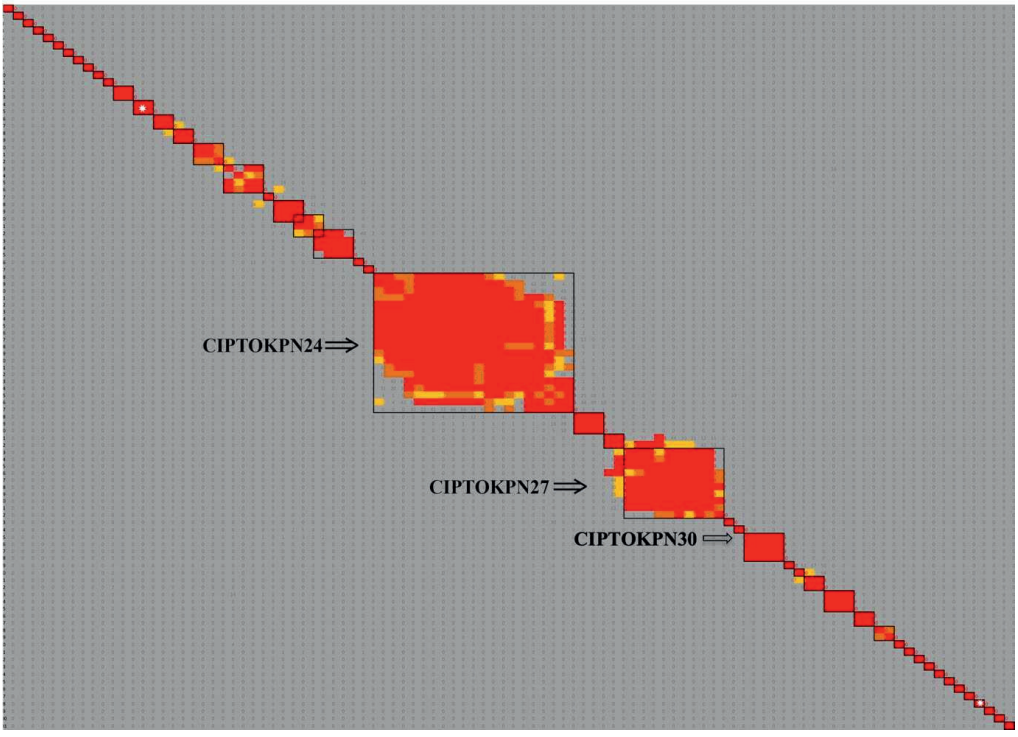
Supplementary Figure 1. *Klebsiella pneumoniae* carriage of included patients admitted to the ICUs (adult- and ER-ICU) of Dr. Cipto Mangukusomo General Hospital, Jakarta, Indonesia



Supplementary Figure 2. Plot of the cumulative incidence for ICUs discharge alive and death by carbapenem-susceptible and -non-susceptible *K. pneumoniae*.

Legend: Abbreviation: ICU, Intensive Care Unit; KP, *Klebsiella pneumoniae*

The probability of being discharged alive (solid lines) and in-ICUs mortality (dashed lines) among patients harboring carbapenem-susceptible *K. pneumoniae* (black lines) versus patients harboring carbapenem-non-susceptible *K. pneumoniae* (red lines) during their ICU stay. The probability of death in ICUs is significantly higher (p=0.006) among patients with carbapenem-non-susceptible *K. pneumoniae*, conversely the probability of being discharged alive from ICUs is significantly higher for patients with carbapenem- susceptible *K. pneumoniae* (p=0.0005)



Supplementary Figure 3. Raman spectroscopy-based cluster analysis of *Klebsiella pneumoniae* isolates from adult and ER-ICUs

Legend: Raman spectra correlation matrix of carbapenem-non-susceptible *K. pneumoniae* isolates. The similarity between pairs of spectra was calculated using the squared Pearson correlation coefficient (R^2-values), multiplied by 100 and expressed as a percentage. The similarity threshold for this study was set at 91% (yellow area) so that two isolates with an R^2 below this threshold were considered to be different and were designated different Raman types. Two isolates with an R^2-value above 99.5% (red area) were considered indistinguishable and were considered to have the same Raman type. In case of an R^2-value between of 91% and 99.5% (orange area), these isolates were considered highly related but not identical. There were three dominant cluster CIPTOKPN24, CIPTOKPN27, and CIPTOKPN30.

CHAPTER 5

5

The epidemiology and characterization of carbapenem-non-susceptible *Pseudomonas aeruginosa* in a large intensive care unit in Jakarta, Indonesia

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International Journal of Antimicrobial Agents 54 (2019) 655–660

ABSTRACT

Objective:

A prospective observational study was performed to assess the epidemiology of carbapenem-non-susceptible *Pseudomonas aeruginosa* (CNPA) in intensive care units (ICUs) of the national referral hospital in Jakarta, Indonesia.

Materials/methods:

Adult patients consecutively hospitalized for >48 hours in two ICUs of the national referral hospital were included. *P. aeruginosa* from clinical cultures and systematic screening were included. Environmental niches and healthcare workers (HCWs) were also screened. Susceptibility was determined phenotypically and the presence of carbapenemase genes by PCR. Multiple-locus variable number tandem repeat analysis (MLVA) and multilocus sequence typing (MLST) were used for genotyping.

Results: Seventeen out of 412 (4.1%) patients carried CNPA on admission and 34/395 (8.6%) became positive during ICU stay. The acquisition rate was 18/1,000 patient-days at risk. Twelve out of 16 (75.0%) environmental isolates were CNPA. HCWs screened negative. Acquisition of CNPA was associated with longer ICU stay (adjusted hazard ratio: 1.89 [CI_{99}: 1.12-3.13]). Mortality was >40% among patients with CNPA versus <30% among those without CNPA (p=0.019). Of the 119 CNPA isolates, 83 (69.7%) carried a carbapenemase gene, bla_{VIM} (n=36), bla_{IMP} (n=23), and bla_{GES-5} (n=24). Four sequence types (ST) dominated (ST235, ST823, ST446, ST357). Five major MLVA clusters were distinguished, two belonging to ST235, the others to ST823, ST446, and ST357.

Conclusions:

CNPA are introduced into these ICUs and some strains expand clonally among patients and the environment, creating endemic CNPA. VIM-, IMP-, and GES-5 genes are often involved. CNPA acquisition was associated with prolonged ICU stay and may affect ICU survival.

Keywords:

Pseudomonas aeruginosa, microbial drug resistance, carbapenemase, metallo-beta-lactamase, intensive care unit, Indonesia.

INTRODUCTION

Beta-lactam antibiotics are the drugs of choice for treatment of severe infections due to *Pseudomonas aeruginosa*. However, worldwide emergence of carbapenem-non-susceptible *P. aeruginosa* (CNPA) compromises treatment of these infections [1]. Non-susceptibility to carbapenem antibiotics in *P. aeruginosa* is usually due to a combination of mechanisms, including beta-lactamase production, increased efflux pump activity, outer membrane modifications, and production of a carbapenemase as a single potent resistance mechanism; VIM, IMP, and GES-5 are most commonly found around the world [2-4].

Little information exists on the epidemiology of CNPA from Indonesia, the fourth most populous country in the world. In 2011, 21.9% of *P. aeruginosa* strains from the ICU of the Dr. Cipto Mangunkusumo General Hospital, Jakarta, were carbapenem-resistant, and four *P. aeruginosa* isolates contained the bla_{IMP} gene [5] .

The aim of our study was to delineate the clinical and molecular epidemiology of CNPA in two ICUs of the Dr. Cipto Mangunkusumo General Hospital, the national referral teaching hospital.

MATERIALS AND METHODS

Study design

A prospective observational study was performed at the Dr. Cipto Mangunkusumo General Hospital, in Jakarta, Indonesia, from April–October 2013 and from April–August 2014. We conducted this study in the 12-bedded adult ICU and the 8-bedded Emergency Room (ER)-ICU with an average of 1,010 and 415 admissions per year, respectively. Both ICUs are open-plan wards [6].

All adult patients (≥18 years old) admitted to those ICUs and hospitalized for more than 48 hours were eligible for enrolment. The first screening cultures were taken on the day of admission. Informed consent was obtained from the patient or their relatives. Demographic and clinical characteristics (age, gender, medical or surgical indication, underlying diseases, hospitalization history, previous use of antibiotics) were recorded on admission. The systemic inflammatory response syndrome (SIRS) and quick Sequential Organ Failure Assessment (qSOFA) score on admission were calculated [7].

The primary outcome measure was acquisition of a CNPA during ICU stay 48 hours after admission. Acquisition of CNPA was defined by a screening culture or clinical culture positive with CNPA. Secondary outcome measures were length of ICU stay, and in-ICU mortality.

Environmental samples were taken twice (in October 2013 and December 2014), in both ICUs. Screening of healthcare workers (HCWs) was performed once (September 2013). HCWs were defined as all personnel (doctors, nurses, cleaning staff, administration staff, porters, nutritionist) working in the ICUs during the study.

Screening cultures were obtained from throat and rectum or stools on the day of admission, at the time of discharge from the ICU, and weekly if the patient's stay exceeded seven days. Sampling was performed using sterile cotton-tipped swabs which were transported in Amies transport medium (Oxoid, UK) at ambient temperature to the laboratory on the same day. All swabs were processed within 24 hours.

Clinical samples were collected on indication under aseptic precautions from the lower respiratory tract, blood, urine, tissue, or wounds.

Environmental samples were taken from various sites (Supplementary Table 1) with swabs and placed in Amies transport medium [6]. From HCWs, throat and rectal swabs were collected.

Microbiological methods

Isolation and identification

In the Clinical Microbiology Laboratory (Faculty of Medicine, Universitas Indonesia, Jakarta) swabs were placed in 5 ml trypticase soy broth (TSB) with cefotaxime 2 mg/L, vancomycin 50 mg/L and incubated overnight. A loop of broth (10 μl) was aerobically sub-cultured onto MacConkey agar (Oxoid, UK) for 16-24 hours, following identification and susceptibility testing of colonies suggestive of *P. aeruginosa* using the VITEK2® (bioMérieux, France).

Blood cultures were collected in BACTEC® (BD, Franklin Lakes, NJ, USA) bottles as per manufacturer's instructions. Other clinical specimens were inoculated onto blood and MacConkey agar plates and incubated as above. All colonies were examined by Gram stain and identified using the VITEK2®.

Strains were stored at -80°C in TSB with 10% v/v glycerol. The identity of strains was confirmed in Erasmus MC, Rotterdam, the Netherlands, by using matrix-assisted laser desorption/ionisation mass spectrometry (Maldi Biotyper, Bruker, UK).

Antimicrobial susceptibility testing

Susceptibility of the screening isolates to imipenem and meropenem was investigated by Kirby-Bauer disk diffusion using Mueller-Hinton plates (BD, USA). For the isolates from clinical cultures, minimum inhibitory concentrations (MICs) were determined by VITEK2®. Carbapenem zone sizes and MICs were interpreted according to EUCAST (2013, breakpoints for non-susceptibility: meropenem <24 mm and MIC >2 mg/L, imipenem <20 mm and MIC >4 mg/L) [8].

Phenotypic detection of carbapenemases

For detection of metallo-beta-lactamase (MBL), the imipenem and doripenem combo disk test with EDTA was performed [9].

DNA extraction and carbapenemase gene detection

DNA was extracted using the InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA). PCRs for Ambler class B MBLs (bla_{NDM}, bla_{VIM}, bla_{IMP}) were carried out using a T3000 Thermocycler (Biometra-Whatman, Germany)[10, 11].

Clonal relatedness

Multiple-locus variable number tandem repeat analysis (MLVA) and *in silico* multilocus sequence typing (MLST) were used for typing with primers according to Supplementary Table 2.[12] Two µL of 100x diluted PCR products were analyzed on an ABI3130xl Genetic Analyzer (Thermo Fisher Scientific, USA). Electropherograms were analyzed using the MLVA plugin in BioNumerics v7.6 (Applied Maths, Belgium). Typing data were analyzed categorically.

MLST types as well as carbapenemase gene subtypes were inferred from whole genome sequencing (WGS) using the MLST plug-in and sequence extraction tool from BioNumerics® v7.6 (Applied Maths, Belgium). The classical 7-digit *in silico* MLST profiles were obtained through BLAST using the pubMLST database hosted at https://pubmlst.org. Sequencing was performed using a HiSeq 2500 instrument (Illumina Inc., USA).

Statistical analysis

Statistical analyses were done using SPSS Version 24.0 (SPSS, USA). Patients admitted to adult ICU were compared to ER-ICU using Chi-square or Fishers' exact and Mann-Whitney as appropriate. One-way ANOVA was used to compare patient characteristics according to their *P. aeruginosa* status. Univariate and multivariate analyses were performed to establish risk factors associated with mortality using a multivariate logistic regression model with backward selection and inclusion of variables with a p-value <0.1 in the univariate analysis. Cox proportional regression was used to analyse risk factors for length of stay. Kaplan-Meier method was performed to construct survival curves. P-values of less than 0.01 were considered significant [13].

RESULTS

Patient characteristics and outcomes

During the study, 1,211 patients were hospitalized in the ICUs (Adult ICU: 863, ER-ICU: 348). Of 412 included patients, 188 were admitted to the adult ICU and 224 to the ER-ICU. Most of the non-eligible patients were excluded due to short length of stay. Comparison of patients' baseline characteristics showed that there were no significant differences between patients in both ICUs, except that in the adult ICU most of the patients had been referred from another ward

and more patients had malignancies (Supplementary Table 3). Therefore, we analysed the data from the ICUs both separately and pooled.

Overall, 145/412 (35.2%) patients had at least one positive culture with *P. aeruginosa*, the remaining 267 patients were free from *P. aeruginosa* on admission and remained so during their ICU stay. Eigthty-three patients (20.2%) already carried *P. aeruginosa* as revealed by cultures taken on the day of admission, of whom 66 patients carried a carbapenem–susceptible *P. aeruginosa* (CSPA) and 17 patients carried a CNPA (Supplementary Figure 1). Thirty-four patients acquired a CSPA, 34 patients acquired a CNPA. For the 51 patients with CNPA, 35 CNPA were obtained from screening specimens, 6 from clinical specimens and 10 from both screening and clinical samples; there were no CNPA-positive blood cultures.

The dynamics of acquisition of *P. aeruginosa* in the ICUs is shown in Figure 1A. Patients who acquired a CSPA had their first positive culture approximately two days earlier than patients that acquired a CNPA (p=0.065). The acquisition rate of CSPA was 19 per 1,000 patient-days at risk (adult ICU: 22; ER-ICU: 14) compared to 18 per 1,000 patient-days at risk for CNPA (adult ICU: 15; ER-ICU: 21).

Patient outcomes were associated with *P. aeruginosa* status. Patients who acquired CNPA had a significantly longer length of stay (median [interquartile range (IQR)]: 16 [6-27] days, adjusted hazard ratio [aHR]: 1.89 [99% confidence interval (CI_{99}): 1.12-3.13], p=0.002, Supplementary Table 4, Figure 1B) compared to the other groups of patients, of whom ≥80% were discharged from the ICU within 7-12 days. These latter groups included the patients that were always free from *P. aeruginosa*, and patients that already carried *P. aeruginosa* (either CSPA or CNPA) at time of ICU admission and patients who became positive for CSPA during their stay in ICUs (Figure 1B).

A longer length of stay was also independently associated with mechanical ventilation ≥5 days (median [IQR]: 10 [7-15], aHR: 3.09 [CI_{99}]: 1.98-4.83], p<0.001, Supplementary Table 4) and use of urine catheters ≥5 days (median [IQR]: 8 [5-12], aHR: 3.03 [CI_{99}: 1.73-5.30], p<0.001, Supplementary Table 4).

Acquisition of *P. aeruginosa* was not associated with in-ICU mortality, 27.7% of patients that remained free of *P. aeruginosa* died versus 14.7% and 41.2% of patients that acquired a CSPA or CNPA, respectively, during (Supplementary Table 5, adjusted Odds Ratio [aOR]: 0.41 [CI_{99}: 0.10-1.71], p=0.109 and 1.08 [CI_{99}: 0.34-3.46], p=0.867). The group of patients that acquired CSPA had the lowest mortality rate, and when compared to those that acquired a CNPA, the probability of ICU survival was higher for patients who acquired a CSPA compared to patients who acquired CNPA (aHR: 4.06 [CI_{99}: 0.87-18.88], p=0.019, Figure 1C). Likewise, the ICU mortality among all patients with CNPA was 22/51 (43.1%) versus 97/361 (26.9%) among patients without CNPA (p=0.016).

The admission SIRS and qSOFA scores of patients with or without *P. aeruginosa* acquisition did not differ, indicating that a significant difference in the risk of dying was not present at the time of ICU admission but rather emerged later during their ICU stay (SIRS: crude Odds Ratio [cOR]: 1.69 [CI_{99}: 0.55-5.22], p=0.230; qSOFA: cOR: 1.45 [CI_{99}: 0.68-3.08], p=0.211, Supplementary Table 5). In multivariate comparison, patients that acquired a CNPA during ICU stay were more likely to have had prior exposure to antibiotics, especially carbapenems (aOR: 2.67 [CI_{99}: 0.94-7.62], p=0.015).

Phenotypic and molecular characterization of CNPA

Overall, 107/281 (38.1%) isolates from 51/145 patients were found to be non-susceptible to either imipenem and/or meropenem. Additionally, 12/16 (75.0%) *P. aeruginosa* isolates cultured from the environment (tap water, dish dryer, table top, water from siphon and water from suction connector) were CNPA (Supplementary Table 6). None of 25 *P. aeruginosa* isolates from HCWs were found to be CNPA. A total of 119 CNPA were subjected to phenotypic and molecular analyses. The phenotypic detection test indicated that 68/119 (57.1%) isolates produced an MBL. PCR demonstrated the presence of bla_{VIM} in 36 of these isolates and bla_{IMP} in 23, including isolates from patients and one from the environment. None of the 119 isolates were positive for bla_{NDM}. Presence of bla_{GES-5} (non-MBL) was deduced from WGS and detected in 24 isolates.

Clonal relatedness

MLST revealed four major clusters (ST235, ST823, ST446, ST357) as well as several new sequence types (STs). By MLVA, five major clusters were distinguished, two belonging to ST235; the other MLVA clusters corresponded with ST823, ST446 and ST357 (Figure 2A). These four major genetic clusters included 97/107 (90.7%) CNPAs from patients (ICU-imported and ICU-acquired) as well as 11/12 (91.7%) environmental isolates (ST235, ST823, ST446) (Figure 2B). Most isolates belonged to ST235 (10 imported and 32 acquired patient isolates, and 4 environmental isolates) and 22 isolates harboured bla_{IMP}, twenty-four isolates harboured bla_{GES-5} but no isolates contained bla_{VIM}. All ST823 isolates (36 isolates) harboured bla_{VIM} (Supplementary Table 6-7).

Table 1. Patient characteristics and outcomes according to *Pseudomonas aeruginosa* status

	Group 1	Group 2	Group 3	Group 4	Group 5	p value
	267	60	17	34	34	
Age (years), median (IQR)	46 (31-58)	44 (32-56)	43 (27-58)	49 (33-60)	47 (37-55)	0.895
Gender (%)						0.530
Male	137 (51.3)	27 (45.0)	11 (64.7)	20 (58.8)	19 (55.9)	
Female	130 (48.7)	33 (55.0)	6 (35.3)	14 (41.2)	15 (44.1)	
Underlying diseases (%)						
Cardiovascular						0.851
Yes	16 (6)	4 (6.7)	1 (5.9)	3 (8.8)	1 (2.9)	
No	251 (94)	56 (93.3)	16 (94.1)	31 (91.2)	33 (97.1)	
Cerebrovascular						0.006
Yes	12 (4.5)	6 (10.0)	0 (0)	4 (11.8)	7 (20.6)	
No	255 (95.5)	54 (90.0)	17 (100.0)	30 (88.2)	27 (79.4)	
Chronic kidney diseases						0.130
Yes	13 (4.9)	6 (10.0)	3 (17.6)	2 (5.9)	1 (2.9)	
No	254 (95.1)	54 (90.0)	14 (82.4)	32 (94.1)	33 (97.1)	
Diabetes mellitus						0.262
Yes	24 (9)	1 (1.7)	1 (5.9)	3 (8.8)	4 (11.8)	
No	243 (91)	59 (98.3)	16 (94.1)	31 (91.2)	30 (88.2)	
Malignancy						0.379
Yes	74 (27.7)	17 (28.3)	6 (35.3)	14 (41.2)	7 (20.6)	
No	193 (72.3)	43 (71.7)	11 (64.7)	20 (58.8)	27 (79.4)	
Indication for ICU admission (%)						0.005
Medical	78 (29.2)	22 (36.7)	9 (52.9)	11 (32.4)	20 (58.8)	
Surgical	189 (70.8)	38 (63.3)	8 (47.1)	23 (67.6)	14 (41.2)	

	Group 1	Group 2	Group 3	Group 4	Group 5	p value
	267	60	17	34	34	
Referral from (%)						0.120
Other ward this hospital	141 (52.8)	26 (43.3)	10 (58.8)	25 (73.5)	20 (58.8)	
Other hospital	46 (17.2)	15 (25.0)	3 (17.6)	4 (11.8)	9 (26.5)	
Directly from Emergency Unit	80 (30)	19 (31.7)	4 (23.6)	5 (14.7)	5 (15.2)	
Antibiotic exposure (pre-ICU admission)						
Any antibiotic (%)	189 (70.8)	52 (86.7)	12 (70.6)	28 (82.4)	30 (88.2)	0.020
Carbapenem (%)	41 (15.4)	9 (15.0)	8 (47.1)	4 (11.8)	17 (50.0)	0.000**
SIRS Score, (%)						0.530
Score ≥2	240 (89.9)	56 (93.3)	17 (100.0)	31 (91.2)	33 (97.1)	
Score <2	27 (10.1)	4 (6.7)	0 (0)	3 (8.8)	1 (2.9)	
qSOFA Score, (%)						0.014
Score ≥2	205 (76.8)	51 (85.0)	17 (100.0)	29 (85.3)	32 (94.1)	
Score <2	62 (23.2)	9 (15.0)	0 (0)	5 (14.7)	2 (5.9)	
Procedures (during ICU admission)						
Mechanical ventilation (%)	233 (87.3)	55 (91.7)	17 (100.0)	33 (97.1)	33 (97.1)	0.126
Mechanical ventilation (days) median (IQR)						0.000**
≥5 days	99 (37.1)	28 (46.7)	13 (76.5)	17 (50.0)	25 (73.5)	
<5 days	168 (62.9)	32 (53.3)	4 (23.5)	17 (50.0)	9 (26.5)	
Central venous catheter (%)	230 (86.1)	52 (86.7)	17 (100.0)	31 (91.2)	33 (97.1)	0.196
Central venous catheter (days) median (IQR)						
≥5 days	125 (46.8)	33 (55.0)	13 (76.5)	23 (67.6)	29 (85.3)	
<5 days	142 (53.2)	27 (45.0)	4 (23.5)	11 (32.4)	5 (14.7)	
Urine catheter (%)	267 (100.0)	60 (100.0)	17 (100.0)	34 (100)	34 (100.0)	N/A

	Group 1	Group 2	Group 3	Group 4	Group 5	p value
	267	60	17	34	34	
Urine catheter (days) median (IQR)						0.001
≥5 days	148 (55.4)	35 (58.3)	13 (76.5)	25 (73.5)	30 (88.2)	
<5 days	119 (44.6)	25 (41.7)	4 (23.5)	9 (26.5)	4 (11.8)	
Antibiotic therapy (during ICU admission)						
Any antibiotic (%)	263 (98.5)	59 (98.3)	16 (94.1)	34 (100.0)	34 (100.0)	0.460
Carbapenem (%)	114 (42.7)	31 (51.7)	14 (82.4)	12 (35.3)	28 (82.4)	0.000**
Outcomes						
Length of stay (days), median (IQR)	4 (3-7)	5 (3-10)	6 (4-12)	7 (3-12)	15 (6-26)	0.000**
Death (%)	74 (27.7)	18 (30.0)	8 (47.1)	5 (14.7)	14 (41.2)	0.064

Abbreviations: ICU, Intensive Care Unit; IQR, Interquartile range; NS, non-susceptible; qSOFA, quick Sepsis-related Organ Failure Assessment; SIRS, Systemic Inflammatory Response Syndrome; S, susceptible.

Group 1: No *P. aeruginosa* on admission and negative for *P. aeruginosa* during ICU admission.

Group 2: Carbapenem-S *P. aeruginosa* on admission, no carbapenem-NS *P. aeruginosa* acquisition during ICU admission.

Group 3: Carbapenem-NS *P. aeruginosa* on admission, considered as positive during ICU admission* (* regardless of results of follow-up cultures).

Group 4: No *P. aeruginosa* on admission, acquisition of carbapenem-S *P. aeruginosa* during ICU admission.

Group 5: Either no *P. aeruginosa* or carbapenem-S *P. aeruginosa* on admission, acquisition of carbapenem-NS *P. aeruginosa* during ICU admission.

Significance was calculated using One-way ANOVA, Pearson Chi Square and Fisher's Exact Test.

A p-value less than 0.01 was considered statistically significant.

**p<0.00

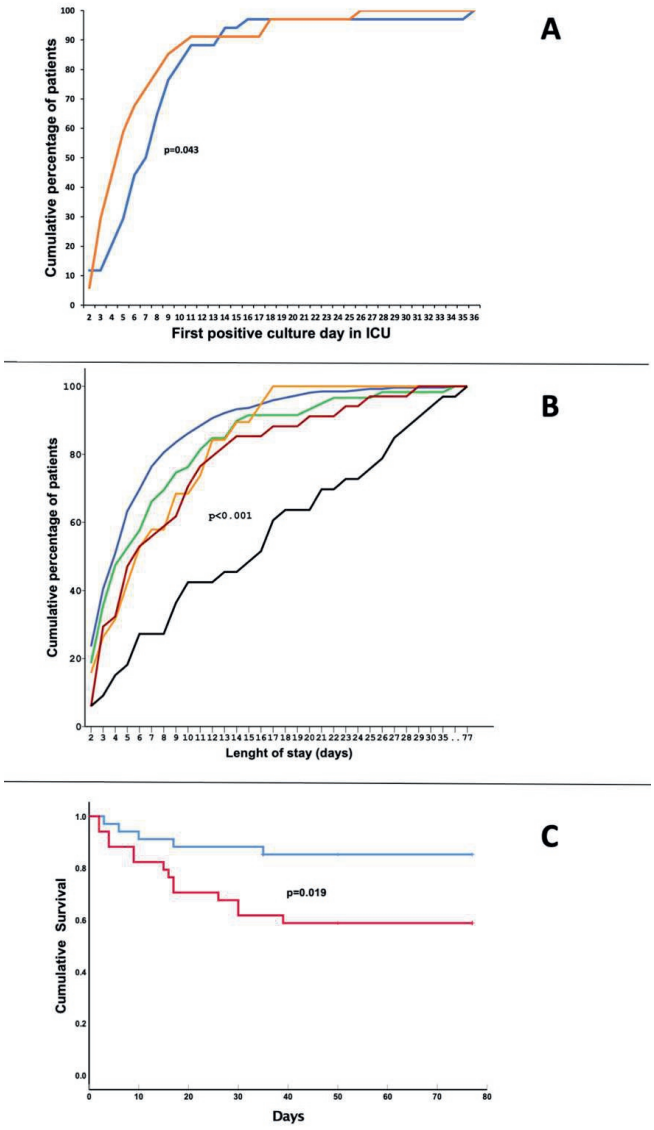

Figure 1. Acquisition of carbapenem-susceptible and –non-susceptible *P. aeruginosa* and its effect on ICU stay and survival. **A,** Acquistion of carbapenem-susceptible *P. aeruginosa* (orange) and carbapenem-non-susceptible *P. aeruginosa* (blue) (P value by independent Mann-Whitney U test). **B,** Length of ICU stay by *P. aeruginosa* status: patients that were always *P. aeruginosa* negative (blue), patients already positive for carbapenem-susceptible *P. aeruginosa* on admission (green), patients already positive for carbapenem-non-susceptible *P. aeruginosa* on admission (yellow), patients that acquired carbapenem-susceptible *P. aeruginosa* during ICU stay (red) and patients that acquired carbapenem-non-susceptible *P. aeruginosa* during ICU stay (black) (P value by Cox regression), and **C,** survival of patients acquiring a carbapenem-susceptible *P. aeruginosa* (blue) compared with patients acquiring a carbapenem-non-susceptible *P. aeruginosa* (red) during their ICU stay (P value by binary regression).

DISCUSSION

The dissemination of *P. aeruginosa* isolates harbouring carbapenemase genes, continues unabated, and reports describing these isolates are emerging from different parts of the world, including Southeast Asia [14]. We found that 12.4% of the patients in our ICUs carried CNPA and 4.1% of patients were already colonized prior to admission. Patients may become colonized elsewhere in the same hospital, or in another hospital from which they are referred, or may come with such strain directly from the community, possibly having acquired their strain during a previous healthcare contact or indirectly from exposure to relatives carrying such strains or unknown environmental niches.

Hence, screening cultures must be performed for early detection and infection control. This will guide rational antibiotic use, since it has been shown that colonization with CNPA is a risk factor for infection [15].

Our data show that patients acquire CNPA during ICU stay and that this is associated with significantly longer ICU stay. At the level of significance chosen, the acquisition of CSPA or CNPA was not associated with mortality when compared to patients free of *P. aeruginosa*. Still, the observed mortality rate was higher among patients with CNPA versus those without. A study in Taiwan (2016) did not find carbapenem resistance as a significant factor associated with mortality either [16]. Other study revealed a relationship between CNPA carriage and mortality [17].

Among the 107 CNPA from 51 patients and 12 environmental niches, bla_{VIM}, bla_{IMP}, and $bla_{\text{GES-5}}$ were the most prevalent carbapenemase genes, detected in 83 isolates, including six from the environment. These genes are widely distributed in the world. The first MBL found in *P. aeruginosa* was IMP-1, originally identified in Japan in 1988. VIM was first identified in Italy in 1997, but reached Southeast Asia [3], [14]. GES-5-producing *P. aeruginosa* was first detected in China in 2004 and was since then isolated on a global scale [18], [19].

In a review by Voor In 't Holt et al., it was shown that carbapenem use and use of medical devices are the leading risk factors for carriage of CNPA [20]. They identified environmental sources and reservoirs with sinks being the most frequently reported reservoirs [21]. More recent reports of outbreaks of CNPA also demonstrate an association with environmental contamination [22-24]. This phenomenon was observed in our setting as well. The four environmental bla_{IMP} -positive ST235 isolates were all cultured from wet sources in the common cleaning room. In this room, located adjacent to the adult ICU, all items that are reused in patients, are manually cleaned and stored. These types of 'wet' rooms may serve as a persistent source of resistant bacteria and should be targeted by infection control.

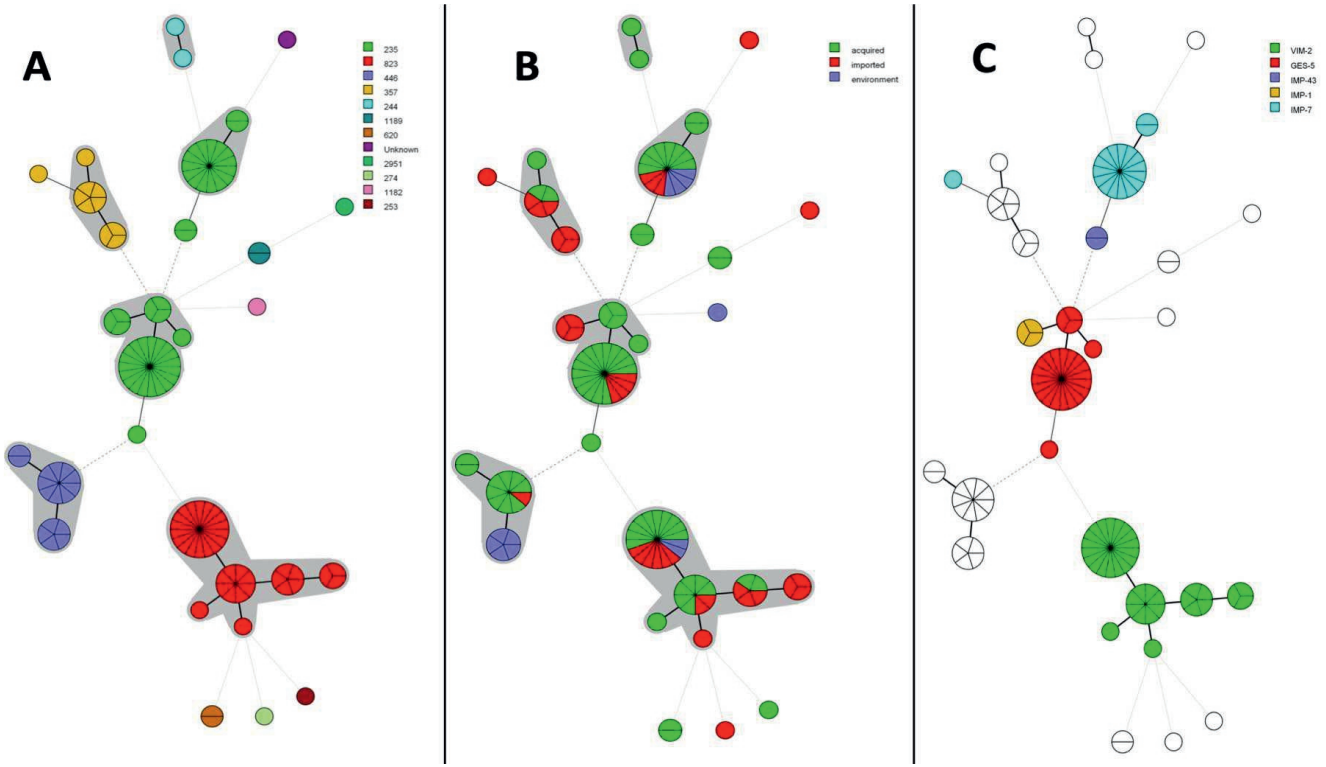

Figure 2. Minimum spanning tree analysis of carbapenem-non-susceptible *P. aeruginosa* isolates based on multiple loci VNTR analysis (MLVA). Grey shading in each panel indicates MLVA complexes. **Panel A**: Six most prevalent MLVA clusters corresponded to ST235 (green), ST823 (red), ST446 (purple), ST357 (yellow), ST244 (light blue) as determined by MLST. Minor clones indicated by other colours in this panel. **Panel B**: Red segments indicate *P. aeruginosa* isolates that were imported into the ICU by patients, green those that were acquired in the ICU by patients and purple segments isolates from the ICU environment. **Panel C**: Coloured segments indicate the carbapenemase genes VIM-2 (green), GES-5 (red), IMP-7 (light blue), IMP-1 (yellow) and IMP-43 (purple). Colourless segments represent isolates without carbapenemase genes.

MLST revealed four major clusters (ST235, ST823, ST446, ST357) and a few new clones, MLVA revealed five major clusters. ST235 is the most prevalent of these so-called 'international' clones associated with poor clinical outcomes in part due to multi- and high-level antibiotic resistance [25]. The Indonesian offspring of this clone, identified in this study, always harboured bla_{IMP} or bla_{GES-5}, but not bla_{VIM}. All ST823 isolates consistently harboured only bla_{VIM} and no MBL genes were found in the other prevalent clones (ST446, ST357). Three dominant clusters included isolates from the ICU environment (ST235, ST823, ST446). This epidemiologic information should be used when designing interventions to reduce the acquisition of CNPA in ICUs in Indonesia and similar settings elsewhere.

Our study has limitations. First, it was a single-centre study during a situation of endemic CNPA colonization and infection, so our data are not representative for the whole country. Second, we were unable to evaluate the effect of other possible confounders of CNPA acquisition (e.g. long-term kidney dialysis, need for inotropes, surgery, previous hospital admission).

CONCLUSIONS

This study is the largest describing characteristics, epidemiology, and outcome of CNPA in ICUs in Indonesia. Colonization or infection with CNPA during ICU hospitalization was independently associated with prolonged length of stay, and possibly survival. ST235 was the dominant clone, as were IMP and VIM MBLs and GES-carbapenemase. Prevention of colonization and infection by these strains would require active screening for carriers among newly admitted patients and for reservoirs within the ICU environment.

DECLARATIONS

Acknowledgements

We are thankful to The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, Dean of Faculty of Medicine Universitas Indonesia, Board of Directors of Dr. Cipto Mangunkusumo General Hospital Jakarta Indonesia, Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands and Critical Care Division, Department of Anesthesia and Intensive Care, Faculty of Medicine Universitas Indonesia / Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia.

Funding

1. **Yulia Rosa Saharman** is an awardee of the DIKTI-NESO Scholarship by The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, and Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands.
2. **Andreu Coello Pelegrin** received funding from the European Union's Horizon 2020 research and innovation programme "New Diagnostics for Infectious Diseases" (ND4ID) under the Marie Skłodowska-Curie grant agreement N° 675412.

Competing interests

ACP, AvB and CM are employees of bioMérieux, a company developing, marketing and selling tests in the infectious disease domain. The company had no influence on the design and execution of the clinical study neither did the company influence the choice of the diagnostic tools used during the clinical study. The opinions expressed in the manuscript are the author's which do not necessarily reflect company policies.

Ethics and Regulatory Considerations

Ethical Approval

The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the research on 17th September 2012, No: 561/PT02.FK/ETIK/2012, (No: 757/UN2.F1/ETIK/X/2014).

Material Transfer Agreement (MTA)

A Material Transfer Agreement (MTA) was reviewed and approved by the Director of National Institute Research and Development, Ministry of Health (No: LB.02.01/I.9.4/8500/2013).

Trial registration.

The study was registered at www.trialregister.nl (No:5541). Candidate number: 23527, NTR number: NTR5541, Date registered NTR: 22nd December 2015

Consent for publication

Informed consent was documented by the use of a written consent form approved by the Ethics Committee Faculty of Medicine Universitas Indonesia/Dr. Cipto Mangunkusumo General Hospital and signed and dated by the subjects/guardians and by the person who conducted the informed consent discussion and two witnesses. The signature confirmed the consent was based on information that had been understood.

Authors' contributions

YRS, AK, HAV, and JAS conceived the study and participated in design of the study. YRS, RS, and DA participated in acquisition of data. YRS, ACP, WHFG, CHWK, AvB, CM, HAV, and JAS performed data analysis and interpreted the data. YRS, ACP, HAV, and JAS drafted the article. All authors participated in critically revising the draft. All authors read and approved the final manuscript.

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SUPPLEMENTAL MATERIALS

Supplementary Table 1. List of environmental samples

Sample site	Number of samples	
	Adult ICU	ER-ICU
Washbasin on ICUs ward	10	5
Monitor	14	10
Ventilator	15	5
Ambu bag	8	
Stethoscope	10	9
Drawer handle bedside cabinet	21	
Plastic multi-purpose container next to each bed	18	8
Stainless steel container	8	15
Flowmeter	15	
Infusion stand	11	8
Infusion pump	9	6
Bed rails	20	14
Tap water (washbasin on ICU ward)	10	8
Chart paper on bedside cabinet	11	6
Bedside cabinet table	15	11
Cleaning room: washbasin	9	
Cleaning room: sink countertop	3	
Cleaning room: mug	2	
Cleaning room: dish rack	3	
Mattress	5	4
Comb	3	
Water from siphon of washbasin	10	5
Water from mug next to each bed	10	6
Massage oil	5	
Chlorine solution after use	3	
Cleaning wipes	3	4
Wall	3	2
Drawer of bedside cabinet	3	
Water from suction	7	
Suction connector/container	3	
Water from humidifier	1	
Water after cleaning a floor	2	
Floor	2	
Nurse station	1	1

Abbreviations: ICU.Intensive Care Unit.

Supplementary Table 2. Markers and Primers used for the MLVA analysis

Marker	Position in PAO1	Labeled primer sequence (5'>3')	Unlabeled primer sequence (5'>3')	Conc. (µM)	Ref
Multiplex 1					
Pae6A	4131564	(FAM)-AACGGGAATTGTTTCCATGA	GGCCAGGGTTCCGATG	1.0	This study
Pae6C	2735110	(VIC)-CTCTTGAGYCTCGGTCACTC	GCGAACAGCACCAGCAG	0.5	ms207 [1]
Pae6E	1671364	(NED)-CTTGCCCTGGGAAGAGC	AGGGCTTCCAGAACGGTTT	0.5	This study
Pae6F	4541644	(PET)-TCGCAACTGAGCTGGTAATG	CACGCGCACAARGAGAA	0.5	ms209 [1]
Multiplex 2					
Pae6B	417867	(FAM)-AGAGGCGGGCGAAGAAG*	GCAACCCGAGCCTATCCT	0.5	This study
Pae6D	1844904	(VIC)-CGACCTCGACGCCTCT	CGGACGGCGACACTGTT	0.2	ms061 [1]
Pae3A	1408402	(NED)-CAGCACGTGCAGGTAGGC	GCCTGCTGGTCAAYCTCG	0.1	This study
Pae5A	1535425	(PET)-CAGCGCCATTATGGACAAG	GCGGGTTCGCAGGAC	0.5	This study

*The underlined residue is not a match to the target sequence but was introduced in this primer to minimize the potential quenching effect of the adjacent G-residue on the FAM label. FAM. HEX. NED and PET represent fluorescent labels used for detection of the PCR products by capillary electrophoresis. The reference refers to previous use of these marker using alternative amplification primers.[1]

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Supplementary Table 3. Baseline characteristics of patients admitted to the adult and emergency Room (ER) ICUs

	Adult ICU	ER-ICU	p value
Number of patients enrolled	188	224	
Age (years). median (IQR)	49 (38-58)	43 (30-58)	0.041
Gender			
Male (%)	91 (48.4)	123 (54.9)	0.188
Female (%)	97 (51.6)	101 (45.1)	
Underlying diseases	126 (54.8)	104 (45.2)	0.000**
Cardiovascular (%)	12 (6.4)	13 (5.8)	0.806
Cerebrovascular (%)	10 (5.3)	19 (8.5)	0.211
Chronic kidney disease (%)	9 (4.8)	16 (17.1)	0.319
Diabetes mellitus (%)	15 (8)	18 (8)	0.983
Malignancy (%)	80 (42.6)	38 (17)	0.000**
Indication for ICU admission			0.039
Medical (%)	54 (28.7)	86 (38.4)	
Surgical (%)	134 (71.3)	138 (61.6)	
Referral from			0.000**
Other ward this hospital (%)	144 (76.6)	78 (34.8)	
Other hospital (%)	20 (10.6)	57 (25.4)	
Directly from Emergency Unit (%)	24 (12.8)	89 (39.8)	
Antibiotic exposure (before admission to ICU)			
Any antibiotic (%)	146 (77.7)	165 (73.7)	0.349
Carbapenem (%)	40 (21.3)	39 (17.4)	0.321
SIRS Score. (%)			0.992
Score ≥2	172 (91.5)	205 (91.5)	
Score <2	16 (8.5)	19 (8.5)	
qSOFA Score. (%)			0.158
Score ≥2	158 (84.0)	176 (78.6)	
Score <2	30 (16.0)	48 (21.4)	
Procedures (during ICU admission)			
Mechanical ventilation (%)	170 (90.4)	201 (89.7)	0.815
Mechanical ventilation (days). median (IQR)	4 (1.5-9)	3 (2-7)	0.591
≥5 days (%)	87 (26.3)	95 (42.4)	0.431
<5 days (%)	101 (53.7)	129 (57.6)	
Central venous catheter (%)	166 (88.3)	197 (87.9)	0.913
Central venous catheter (days). median (IQR)	5.5 (3-10)	5 (3-8.5)	0.150
≥5 days (%)	106 (56.4)	117 (52.2)	0.400
<5 days (%)	82 (43.6)	107 (47.8)	
Urine catheter (%)	188 (100)	224 (100)	N/A
Urine catheter (days). median (IQR)	6 (3-11)	5 (3-9)	0.181
≥5 days (%)	118 (62.8)	133 (59.4)	0.486

	Adult ICU	ER-ICU	p value
<5 days (%)	70 (37.2)	91 (40.6)	
Antibiotic therapy (during ICU admission)			
Any antibiotic (%)	188 (100)	218 (97.3)	0.034
Carbapenem (%)	99 (52.7)	100 (44.6)	0.105
Outcomes			
Length of stay (days). median (IQR)	5 (3-10.75)	5 (3-8)	0.024
Death (%)	52 (27.7)	67 (29.9)	0.616

Abbreviations: ER-ICU. Emergency room Intensive Care Unit; ICU. Intensive Care Unit; IQR. Interquartile range; qSOFA. quick Sepsis-related Organ Failure Assessment; SIRS. Systemic Inflammatory Response Syndrome.

**p<0.001

Supplementary Table 4. Variables associated with length of stay among patients with and without carbapenem-non-susceptible *Pseudomonas aeruginosa*

	Length of stay Median (IQR)	Univariate analysis				Multivariate analysis			
		p	cHR	99% CI		p	aHR	99% CI	
				Lower	Upper			Lower	Upper
Group									
Group 1	4 (3-7)		1.00				1.00		
Group 2	4 (3-9)	0.075	1.76	0.85	2.55	0.726	0.95	0.65	1.39
Group 3	6 (4-11)	0.262	1.86	0.68	3.54	0.156	0.69	0.36	1.35
Group 4	6 (3-12)	0.018	2.36	1.47	3.79	0.411	1.16	0.72	1.89
Group 5	15 (6-26)	<0.01	4.40	2.68	7.24	0.002	1.89	1.12	3.13
Gender									
Male	5 (3-10)	0.426	0.92	0.72	1.19				
Female	4 (3-9)		1.00						
Underlying diseases									
Cardiovascular									
Yes	8 (5-12)	0.199	1.31	0.77	2.22				
No	5 (3-9)		1.00						
Cerebrovascular									
Yes	8 (3-14)	0.026	1.54	0.93	2.55	0.696	1.09	0.63	1.88
No	5 (3-9)		1.00				1.00		
Chronic kidney diseases									
Yes	4 (3-6)	0.041	0.65	0.38	1.12	0.467	0.85	0.48	1.50
No	5 (3-10)		1.00				1.00		
Diabetes mellitus									
Yes	7 (4-14)	0.108	1.34	0.84	2.14				
No	5 (3-9)		1.00						

	Length of stay Median (IQR)	Univariate analysis				Multivariate analysis			
		p	cHR	99% CI		p	aHR	99% CI	
				Lower	Upper			Lower	Upper
Malignancy									
Yes	4 (3-9)	0.214	0.87	0.66	1.16				
No	5 (3-9)		1.00						
Indication for ICU admission									
Medical	6 (3-12)	<0.001	1.52	1.15	2.01	0.590	1.07	0.79	1.45
Surgical	4 (3-8)		1.00				1.00		
Referral from									
Other ward this hospital	5 (3-10)	0.197	1.21	0.83	1.77				
Other hospital	5 (3-9)	0.151	1.18	0.88	1.59				
Directly from Emergency Unit	5 (3-8)		1.00						
Antibiotic exposure (before admission to ICU)									
Any antibiotic	5 (3-10)	0.014	1.33	0.99	1.79	0.048	1.27	0.93	1.72
No any antibiotic	4 (3-7)		1.00				1.00		
Carbapenem	8 (3-13)	0.012	1.37	0.99	1.90	0.501	1.10	0.77	1.57
No carbapenem	5 (3-8)		1.00				1.00		
SIRS score									
Score ≧2	5 (3-9)	0.651	1.08	0.69	1.71				
Score <2	4 (2-7)		1.00						
qSOFA									
Score ≧2	5 (3-10)	<0.001	1.62	1.17	2.25	0.085	1.26	0.89	1.76
Score <2	3 (2-6)		1.00				1.00		
Procedures (during ICU admission)									
Mechanical ventilation used	5 (3-10)	<0.001	2.50	1.61	3.89	0.351	1.19	0.74	1.93
No Mechanical ventilation used	3 (2-4)		1.00				1.00		

	Length of stay Median (IQR)	Univariate analysis				Multivariate analysis			
		p	cHR	99% CI		p	aHR	99% CI	
				Lower	Upper			Lower	Upper
Mechanical ventilation (days)									
≥5 days	10 (7-15)	<0.001	6.33	4.59	8.73	<0.001	3.09	1.98	4.83
<5 days	3 (2-4)		1.00				1.00		
Central venous catheter used	5 (3-10)	<0.001	2.12	1.42	3.17	0.716	0.93	0.56	1.54
No central venous catheter used	3 (2-5)		1.00				1.00		
Central venous catheter (days)									
≥5 days	9 (6-13)	<0.001	6.30	4.62	8.59	0.052	1.53	0.87	2.67
<5 days	3 (2-3)		1.00				1.00		
Urine catheter used	5 (3-9)	N/A							
No urine catheter used	N/A								
Urine catheter (days)									
≥5 days	8 (5-12)	<0.001	9.87	6.84	14.24	<0.001	3.03	1.73	5.30
<5 days	3 (2-3)		1.00				1.00		
Antibiotic therapy (during ICU admission)									
Any antibiotic (%)	5 (3-9)	0.005	3.20	1.10	9.33	0.335	1.51	0.50	4.49
No any antibiotic	2 (2-3)		1.00				1.00		
Carbapenem (%)	7 (4-12)	<0.001	1.78	1.37	2.32	0.910	0.99	0.71	1.37
No Carbapenem	4 (2-7)						1.00		
Age. correlation coefficient (r)	0.081	0.154	1.00	0.99	1.00	0.550	1.00	0.99	1.01
Mortality	6 (3-12)	0.057	1.23	0.93	1.63	0.094	0.83	0.61	1.10

Abbreviation: aHR, adjusted Hazard Ratio; cHR, crude Hazard Ratio; CI, Confidence Interval; ICU, Intensive Care Unit; IQR, Interquartile Range; LOS, Length of Stay; NS, Nonsusceptible; S, Susceptible; SIRS, Systemic Inflammatory Response Syndrome; qSOFA, quick Sepsis-related Organ Failure Assessment .

Group 1: No *P. aeruginosa* on admission and negative for *P. aeruginosa* during ICU admission.

Group 2: Carbapenem-S *P. aeruginosa* on admission, no carbapenem-NS *P. aeruginosa* acquisition during ICU admission.

Group 3: Carbapenem-NS *P. aeruginosa* on admission, considered as positive during ICU admission* (* regardless of results of follow-up cultures).

Group 4: No *P. aeruginosa* on admission, acquisition of carbapenem-S *P. aeruginosa* during ICU admission.

Group 5: Either no *P. aeruginosa* or carbapenem-S *P. aeruginosa* on admission, acquisition of carbapenem-NS *P. aeruginosa* during ICU admission.

A p-value less than 0.01 was considered statistically significant.

Supplementary Table 5. Variables associated with mortality among patients with and without carbapenem-non-susceptible *Pseudomonas aeruginosa*

			Univariate analysis				Multivariate analysis			
	Mortality		p	99% CI			p	99% CI		
	N	%		cOR	Lower	Upper		aOR	Lower	Upper
Group										
Group 1	74	62.2		1.00						
Group 2	18	15.1	0.722	1.18	0.50	2.50	0.660	0.86	0.35	2.11
Group 3	8	6.7	0.096	2.32	0.63	8.51	0.905	1.07	0.25	4.50
Group 4	5	4.2	0.112	0.45	0.12	1.65	0.109	0.41	0.10	1.71
Group 5	14	11.8	0.108	1.83	0.70	4.79	0.867	1.08	0.34	3.46
Gender										
Male	66	55.5	0.362	1.22	0.70	2.14				
Female	53	44.5		1.00						
Underlying diseases										
Cardiovascular										
Yes	9	7.6	0.418	1.42	0.47	4.31				
No	110	92.4		1.00						
Cerebrovascular										
Yes	13	10.9	0.049	2.12	0.78	5.81	0.572	1.29	0.41	4.03
No	106	89.1		1.00				1.00		
Chronic kidney diseases										
Yes	13	10.9	0.011	2.87	0.98	8.39	0.043	2.60	0.77	8.78
No	106	89.1		1.00						
Diabetes mellitus										
Yes	11	9.2	0.556	1.25	0.46	3.40				
No	108	90.8		1.00						

			Univariate analysis				Multivariate analysis		
	Mortality		p	99% CI			p	99% CI	
	N	%		cOR	Lower	Upper		aOR	Lower	Upper
Malignancy										
Yes	30	25.2	0.326	0.79	0.42	1.48				
No	89	74.8		1.00						
Indication for ICU admission										
Medical	61	51.3	<0.001	2.85	1.59	5.10	0.016	1.81	0.96	3.40
Surgical	58	48.7		1.00				1.00		
Referral from										
Other ward this hospital	58	48.7	0.798	0.94	0.48	1.83	0.197	0.68	0.31	1.48
Other hospital	30	25.2	0.096	1.69	0.75	3.80	0.979	0.99	0.39	2.54
Directly from Emergency Unit	31	26.1		1.00				1.00		
Antibiotic exposure (before admission to ICU)										
Any antibiotic	100	84.0	0.011	2.05	0.99	4.23	0.341	1.36	0.60	3.10
No any antibiotic	19	16.0		1.00				1.00		
Carbapenem	36	30.3	<0.001	2.52	1.29	4.91	0.056	1.62	0.85	3.10
No Carbapenem	83	69.7		1.00				1.00		
SIRS score										
Score ≧2	112	94.1	0.230	1.69	0.55	5.22				
Score <2	7	5.9		1.00						
qSOFA										
Score ≧2	101	84.9	0.211	1.45	0.68	3.08				
Score <2	18	15.1		1.00						
Procedures (during ICU admission)										
Mechanical ventilation used (%)	119	100	N/A							

			Univariate analysis				Multivariate analysis			
	Mortality		p	99% CI			p	99% CI		
	N	%		cOR	Lower	Upper		aOR	Lower	Upper
No Mechanical ventilation used (%)	0	0								
Mechanical ventilation (days)										
≥5 days	74	62.2	<0.001	2.82	1.58	5.02	0.001	5.10	1.46	17.70
<5 days	45	37.8		1.00				1.00		
Central venous catheter used (%)	116	97.5	<0.001	7.20	1.51	34.34	0.006	6.38	1.13	36.09
No central venous catheter used (%)	3	2.5		1.00				1.00		
Central venous catheter (days)										
≥5 days	77	64.7	0.006	1.85	1.04	3.29	0.011	0.28	0.08	1.02
<5 days	42	35.3		1.00				1.00		
Urine catheter used (%)	119	100	N/A							
No urine catheter used (%)	0	0								
Urine catheter (days)										
≥5 days	85	71.4	0.005	1.91	1.05	3.50	0.651	1.29	0.31	5.40
<5 days	34	28.6		1.00				1.00		
Antibiotic therapy (during ICU admission)										
Any antibiotic (%)	119	100	N/A							
No any antibiotic	0	0								
Carbapenem (%)	81	68.1	<0.001	3.16	1.75	5.72	0.005	2.06	1.07	3.97
No Carbapenem	38	31.9		1.00				1.00		
Age. median (IQR) (years old)	50	(38-60)	0.009	1.02	1.00	1.04	0.138	1.01	0.99	1.03
LOS. median (IQR) (days)	6	(3-12)	0.058	1.03	0.99	1.06	0.171	0.98	0.93	1.02

Abbreviation: aOR, adjusted Odds Ratio; cOR, crude Odds Ratio; CI, Confidence Interval; ICU: Intensive Care Unit; IQR, Interquartile range; LOS, Length of Stay; NS, Non-susceptible; S, Susceptible; SIRS, Systemic Inflammatory Response Syndrome; qSOFA, quick Sepsis-related Organ Failure Assessment.

Group 1: No *P. aeruginosa* on admission and negative for *P. aeruginosa* during ICU admission.

Group 2: Carbapenem-S *P. aeruginosa* on admission, no carbapenem-NS *P. aeruginosa* acquisition during ICU admission.

Group 3: Carbapenem-NS *P. aeruginosa* on admission, considered as positive during ICU admission* (* regardless of results of follow-up cultures).

Group 4: No *P. aeruginosa* on admission, acquisition of carbapenem-S *P. aeruginosa* during ICU admission.

Group 5: Either no *P. aeruginosa* or carbapenem-S *P. aeruginosa* on admission, acquisition of carbapenem-NS *P. aeruginosa* during ICU admission.

A p-value less than 0.01 was considered statistically significant.

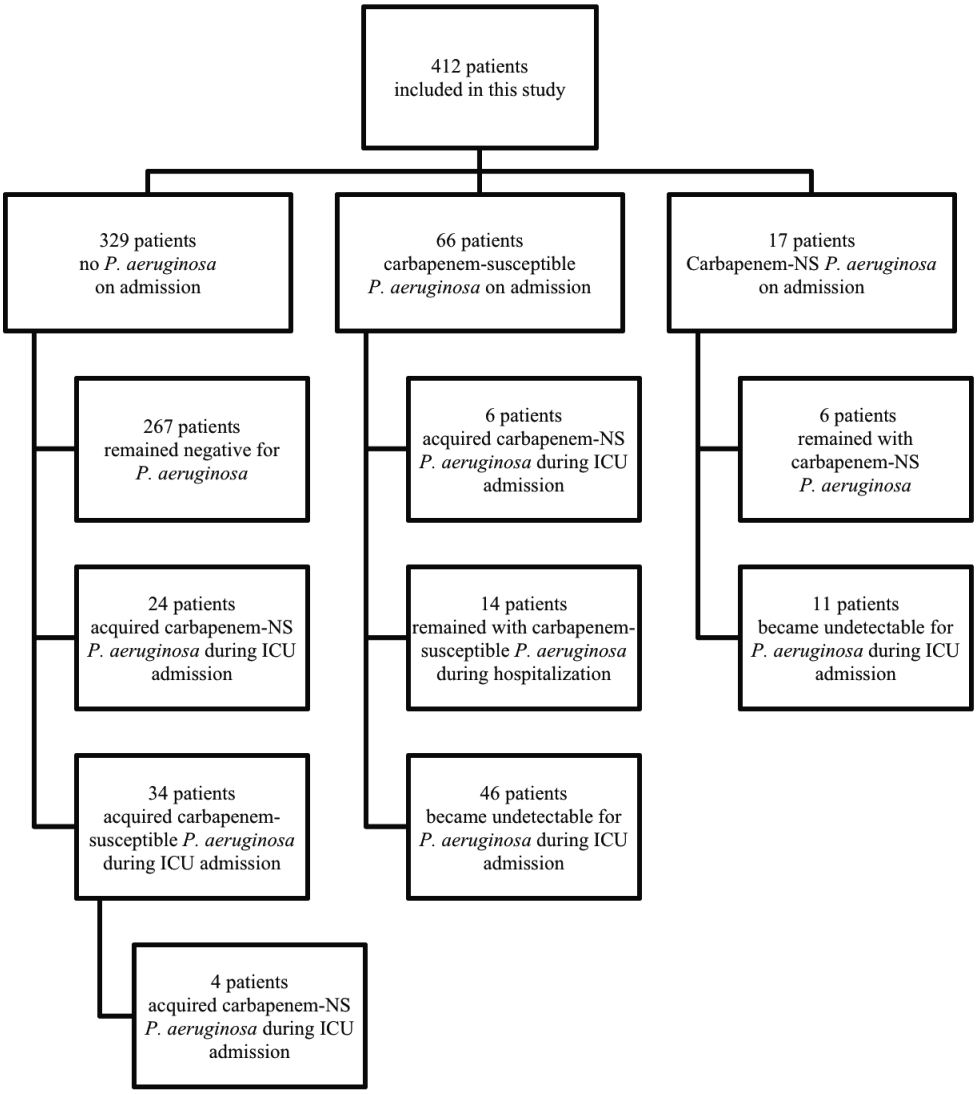
Supplementary Table 6. Origin of carbapenem-non-susceptible *Pseudomonas aeruginosa* from hospital environments.

Origin	ID number	Ward	MLST	Carbapenemase gene
Washbasin bed 2	W 2A ICU 130814	Adult ICU	446	
Washbasin bed 4	W 4C IGD 130814	ER-ICU	1182	
Washbasin bed 5	W 5C IGD 130814	ER-ICU	823	bla_{VIM}
Swab of dish dryer on Cleaning room	8EICU1214(1)	Adult ICU	235	bla_{IMP}
Swab of dish dryer on Cleaning room	8EICU1214(2)	Adult ICU	235	bla_{IMP}
Washbasin 2 on Cleaning room	9EICU1214	Adult ICU	235	bla_{IMP}
Swab of countertop 1 (left) on Cleaning room	3EICU1214	Adult ICU	235	bla_{IMP}
Tap Water from washbasin 1	34EICU1214(1)	Adult ICU	446	
Tap Water from washbasin 1	34EICU1214(2)	Adult ICU	446	
Water from siphon 1	44EICU1214(1)	Adult ICU	446	
Water from siphon 1	44EICU1214(2)	Adult ICU	446	
Water from suction connector bed 2	115EICU1214	Adult ICU	823	bla_{VIM}

Supplementary Table 7. Molecular characterization and clonal relatedness from four major clusters of MLST

ST	No. of isolates "imported" by patients	No. of isolates "acquired" by patients	Environmental isolates	No. of isolates with *bla*$_{VIM}$	No. of isolates with *bla*$_{IMP}$	No. of isolates with *bla*$_{GES-5}$
235	10	32	4		22	24
823	15	19	2	36		
446	1	10	5			
357	7	3			1	

Abbreviations: MLST, Multi Locus Sequence Type; ST, Sequence Type



Supplementary Figure 1. *Pseudomonas aeruginosa* carriage of included patients admitted to the ICUs (adult and emergency room) of Dr. Cipto Mangunkusomo General Hospital, Jakarta, Indonesia

CHAPTER 6

6

Evaluation of whole-genome sequencing-based typing approaches for *Pseudomonas aeruginosa*

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Submitted

ABSTRACT

Pseudomonas aeruginosa is an opportunistic human pathogen, well known for its multi-drug resistance potential, which contributes to nosocomial morbidity and mortality. Whole-genome sequencing (WGS) is key for outbreak detection, but important barriers need to be crossed before WGS can be fully implemented in diagnostic routine microbiology laboratories. Our objective was to evaluate five typing techniques using an epidemiologically well-characterized set of 242 *P. aeruginosa* isolates. DNA was extracted and then sequenced using Illumina® instruments. *In-silico* seven-loci multi-locus sequence typing (MLST) and core and whole genome MLST (cg/wgMLST) were performed using BioNumerics® 7.6 (bioMérieux), while core Single Nucleotide Polymorphism (SNP) analysis (cgSNP) was done with kSNP3. *In-vitro P. aeruginosa*-specific eight-loci Multi-Locus Variable Number of Tandem Repeat (VNTR) Analysis (MLVA) scheme was performed by PCR amplification and size determination of the markers. Additionally, *In-silico* MLVA results were inferred from the WGS data.

Simpson's diversity indices (SDI) and adjusted Wallace (AW) coefficients were calculated to compare between techniques. SDI values showed that most WGS-based typing approaches yield the highest diversity in genotypes, except for the *in-silico* MLST technique (*in-silico* MLST: 0,78; MLVA: 0,89; cgMLST: 0,96; wgMLST: 0,96; cgSNP: 0,93). AW values performed similarly. No statistically significant differences in resolution were found between cgMLST, wgMLST and cgSNP. Our findings show that these three techniques provide the highest level of resolution allowing detailed epidemiological analysis of local outbreaks and international dissemination. MLVA is a suitable alternative for accurate typing of *P. aeruginosa*, useful in settings where the transition towards WGS is currently not feasible.

***Keywords**:*

Pseudomonas aeruginosa, whole genome sequencing, bacterial typing, epidemiology, single nucleotide polymorphism, Indonesia.

INTRODUCTION

Pseudomonas aeruginosa is a gram negative bacterial species found in multiple environments including hospital settings where it may be associated with environmental contamination and complicated infections [1]. *P. aeruginosa* has an extended intrinsic and acquired antimicrobial resistance gene repertoire, and can be found endemically in nosocomial settings, with the intensive care unit (ICU) as a central hot spot [2]. Despite recent successful efforts to associate antibiotic susceptibility phenotypes and certain genomic resistance determinants [3,4], its antimicrobial resistance (AMR) mechanisms arecomplex, and hospital-associated outbreaks caused by *P. aeruginos*a have dramatic repercussions. In order to tackle this problem, infection-control teams use a broad range of typing techniques to help trace bacterial sources, to define the mode of dissemination of pathogenic clones and, ultimately, to hinder or preferably stop microbial spread. Most routine medical microbiology laboratories across the world currently use molecular biological techniques for this purpose, and for the last decades Pulsed-Field Gel Electrophoresis (PFGE; based on the fragmentation of the bacterial genome by a specific enzyme) has been one of the most commonly used techniques in diagnostic laboratories across the world [5]. In addition, Multi-locus variable number of tandem repeats (VNTR) analysis (MLVA) is a frequently used typing technology used to study the epidemiology of *P. aeruginosa* [6]. MLVA is a PCR-based typing technique that takes advantage of VNTRs scattered across a genome. It has certain advantages over other typing techniques such as PFGE or multi-locus sequence typing (MLST, based on polymorphisms in a limited number of housekeeping genes). Despite its current lack of documented inter-centre reproducibility and standardization, MLVA is less expensive and faster than most other typing techniques. In addition, its versatile typing capacities have pushed the development of MLVA-schemes for several microorganisms [7–11].

In *P. aeruginosa*, the first MLVA scheme was proposed by Onteniente *et al*., who evaluated 201 tandem repeat loci to finally select seven of these for the development of typing assays (ms010, ms061, ms077, ms127, ms142, ms172, and ms173) [6]. The scheme has evolved since then, and different approaches with different loci or different VNTR numbers have been evaluated [12–14]. Several studies have proven *P. aeruginosa*-MLVA‘s relevance, for example at tracing the source of environmental *P. aeruginosa* isolates [15], or the longitudinal follow-up of cystic fibrosis (CF) patients infected with *P. aeruginosa* [16].

During the last few years, whole-genome sequencing (WGS) has become an essential tool for global epidemiological surveillance of infectious diseases. A variety of life-science domains is already using WGS, i.e. for taxonomic purposes or in the detection of foodborne pathogen outbreaks [17], and it is expected that it will outperform classic molecular typing techniques as those described above [18,19]. Among all existing WGS-based typing approaches two seem to be more popular than the rest: the use of whole and/or core-genome multi-locus sequence typing

(wg/cgMLST) and the use of whole and/or core-genome Single Nucleotide Polymorphisms analysis (wg/cgSNP) [20,21].

Despite the boom of third and fourth generation WGS technologies and the diagnostic appeal of such nucleotide-precise methodologies, their cost, lack of standardized procedures and software, and practical time-to-result duration negatively affect their implementation in many clinical settings. Therefore, it is important to keep "classical" molecular diagnostic typing technologies such as MLVA readily available and updated, as they still have a significant role to play, even in those laboratories transitioning towards WGS-based typing methods. Obviously, this includes laboratories localized in low-resource settings.

In this paper, we compare different genotyping strategies using over 200 clinical *P. aeruginosa* isolates from an Indonesian ICU setting. Among the methodologies used, we present the wgMLST scheme for *P. aeruginosa* used in BioNumerics® 7.6 (bioMérieux, Sint-Martens-Latem, Belgium). The first objective is to show the resolution-differences among these typing approaches by retrospectively analysing a large collection of clinical *P. aeruginosa*. Secondly, we discuss qualities and practical problems related to the various approaches.

MATERIALS AND METHODS

Bacterial identification and DNA extraction

All isolates were collected and stored during a previously described clinical intervention study [22] and were regrown using Columbia agar + 5% sheep blood. After 18-24h of incubation, single colonies where identified by matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry (MS) (VITEK® MS, bioMérieux, France). DNA was extracted manually using the UltraClean® microbial DNA isolation kit (Qiagen N.V., Venlo, The Netherlands) following the manufacturer's instructions. Further details on the source of the strains and the microbiological methods applied have been presented earlier [22,23].

P. aeruginosa MLVA scheme

The scheme used in this paper has been applied and described in a recent publication regarding the epidemiology of carbapenem non-susceptible *P. aeruginosa* (CNPA) isolates in an Indonesian ICU [22]. The locus values obtained by this previous *in-vitro* MLVA typing effort were imported to BioNumerics® 7.6 (bioMérieux). Additionally, we used the sequence extraction tool to analyze the MLVA markers and their flanking regions from the draft genomes (check Supplementary Table 2 in Saharman, YR 2019 [22]), aligned the resulting sequences and counted the number of tandem repeats, and in this way performed *in-silico* MLVA typing.

Whole-genome sequencing

In brief, the genomes of the 242 isolates were sequenced using either HiSeq2500 or NextSeq500 instruments (Illumina Inc., Cambridge, United Kingdom) using Nextera XT library preparation (Illumina Inc.) generating 150-bp paired-end reads. We used the isolates previously described [23], and an additional set of nine new isolates to complete the collection of Indonesian *P. aeruginosa* strains (details in supplementary **Table S1**). All genomes were assembled using the A5-MiSeq pipeline (v20160825). Assembly quality was verified with QUAST (v5.0.2) [24]. Draft-genomes were imported into BioNumerics®.

Using BioNumerics® we performed *in-silico* MLST; *P. aeruginosa* housekeeping genes *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE* were extracted using the sequence extraction tool and compared to the pubMLST database (hosted at www.pubmlst.org) to obtain the locus identities and sequence types (STs). Starting from the draft genomes, we also used the *P. aeruginosa* wgMLST scheme available in BioNumerics® to identify loci and characterize the corresponding alleles. Details on the wgMLST scheme creation can be found in supplementary **Text S1**. Based on these wgMLST profiles, we described a cgMLST approach valid only for our collection of isolates. Details on how we established the cgMLST are available in supplementary **Text S2**. Finally, a cgSNP analysis was performed with kSNP3 (v3.1) a tool that uses *k*-mers (nucleotides of length *k*) to identify SNPs [25]. kSNP3 was executed using "-k 21-core" parameters. The cgSNP panel, containing the concatenated cgSNPs of all isolates in FASTA format were imported to BioNumerics®.

Data availability

Raw reads and assemblies from sequenced samples are available at the European Nucleotide Archive (ENA) website under the project name PRJEB30625 and PRJEB32907. Additionally, the newly sequenced samples (see supplementary **Table S1**) are available at the ENA under the project name PRJEB35006.

Comparison

In order to evaluate the resolution and congruence of the five typing techniques, we calculated the Simpson's diversity index (SDI) [26] and the adjusted Wallace coefficient (AW) for the clusters obtained by each technique (http://www.comparingpartitions.info/?link=Tool) (accessed on 31st January 2020) 27.. The SDI provides a measure of the resolution at which we can reliably differentiate isolates. The AW is an adjusted version of Wallace coefficient [28], a measure of congruence of two typing methods, which additionally corrects for chance agreement.

For all techniques, we collected information regarding the SDI and WA values, and the optimal cut-off values. To calculate the optimal cut-off values for the different techniques we used

Receiving Operator Characteristics (ROC) curves as previously described [23]. In brief, pairwise matrices of loci or SNP differences exported from BioNumerics and KSNP3 were used to evaluate different thresholds. We assumed that truly identical strains could only be cultured from the same patient, and that different patients never shared the same strain. Based on this we calculated with a custom Python script the number of true positives, true negatives, false positives and false negatives for each threshold, and then calculated their sensitivity and specificity. Using these sensitivity and specificity values we calculated a ROC curve, and set the optimal cut-off using Youden's Index.

RESULTS

Strains

MALDI-TOF MS confirmed that all isolates were correctly identified as *P. aeruginosa* (results not shown).

Typing

The different typing methods were applied to the whole set of 242 *P. aeruginosa* isolates. Loci from the cgMLST scheme were used to generate a Minimum-Spanning Tree (MST) that served as the basis for visualizing the differences in strain clustering between the five typing techniques (**Figure 1**). This information is displayed in **Table 1**. The AW values, which display a quantitative measure of agreement between typing techniques are displayed in **Table 2**. Data will be discussed per technology and in more detail below.

In-silico MLST

Classical MLST revealed the existence of four main MLST sequence types, ST235, ST357, ST823, and ST446 and 20 minor sequence types (each containing less than five isolates). A new sequence type (ST3309) was described among the nine new isolates included in this study. MLST clustering can be observed in **Figure 1A**.

In-vitro and in-silico MLVA

This eight-loci MLVA scheme identified seven main genotypes (MC1 to MC7 in **Figure 1B**; each containing ≥ 10 isolates per cluster) and 20 minor clusters (the rest, containing less than ten isolates per cluster), including 40 singletons. The MP2-PET locus was not present in 158/242 (65.3%) isolates. The correlation of the number of tandem repeats *in-vitro* and *in-silico* can be found in **Figure 2**. We observed a good correlation for six out of eight markers, the less concordant being MP1-NED and MP2-VIC which showed major discrepancies (more than two repeat-unit differences) in 11 and 18 isolates, respectively. In both markers these major

discrepancies were a result of deviations in the *in-silico* approach, underestimating the total number of repeat-units. Consequently, the *in-silico* MLVA detected two genotypes less (*n*=35) than the *in-vitro* MLVA (*n*=37) and had a slightly lower SDI value (see Table 1).

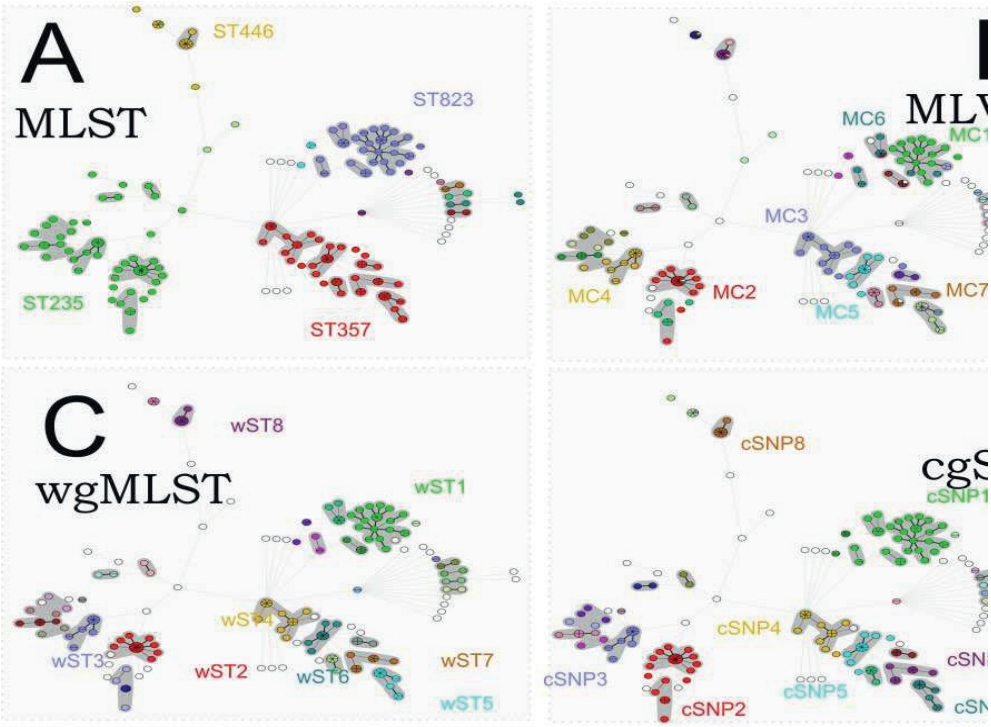

Figure 1. Minimum spanning trees (MSTs) obtained from the *in-vitro* cgMLST analysis character data, as displayed by BioNumerics. Each circle represents a unique cgMLST genotype, the number of isolates with that genotype is represented as sectors of the circles. MSTs A through D were obtained from the different typing techniques, colors represent unique genotyes identified by the corresponding technique. There is no correlation whatsoever between the colors of the different figures. White circles represent singletons. A) *in-silico* MLST; B) *in-vitro* MLVA; C) whole-genome MLST; D) core-genome SNPs.

Table 1. Outputs of the typing techniques evaluated

Technology	# loci	# SNP	cut-off [a]	# of genotypes	SDI (95% confidence interval)
***in-vitro* MLVA**	8	NA	≤ 1	37	0,885 (0.867-0.903)
***in-silico* MLVA**	8	NA	≤ 1	35	0,864 (0.844-0.885)
***in-silico* MLST**	7	NA	0	24	0,782 (0.755-0.810)
cgMLST	4342	NA	≤ 5	68	0,960 (0.951-0.969)
wgMLST	9350	NA	≤ 11	67	0,959 (0.951-0.968)
cgSNP	NA	72016	≤ 4	53	0,930 (0.915-0.946)

SDI, Simpson's diversity index; SNP, Single nucleotide polymorphism.

[a] calculated as described in Materials and methods, and previously in [23];

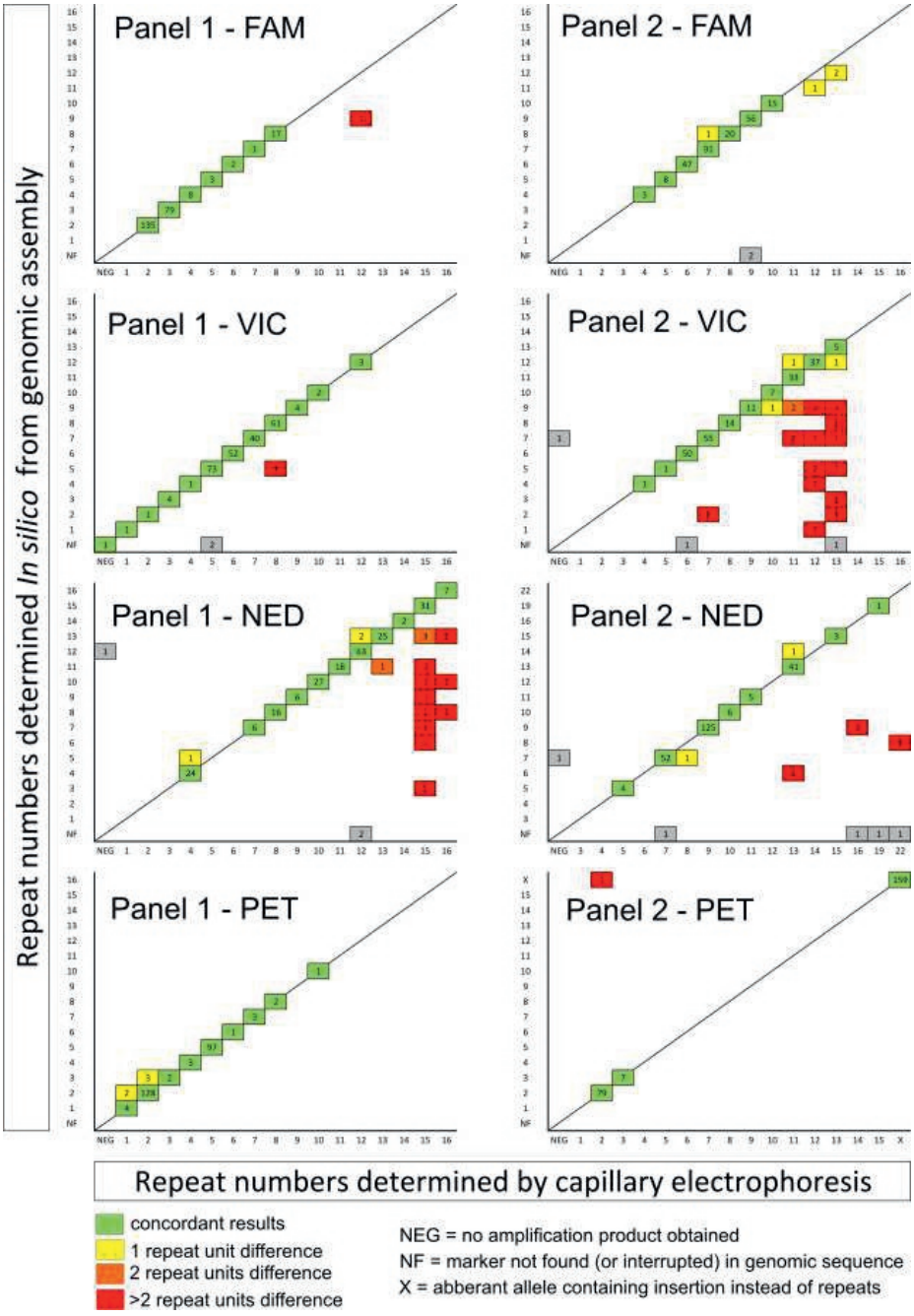

Figure 2. Agreement between the number of repeat units obtained *in-vitro* and *in-silico*. Each locus of the MLVA scheme is represented in a panel. In each panel, the boxes represent the agreement between the repeat units obtained from the two approaches, as described in the colored legend. Additionally, the number inside each box represent the number of isolates involved.

cgMLST

The core genome is usually defined as the set of genes present in all isolates of a species. The number of such common genes present in all strains of our collection was 2,527, but this number doubled when the definition of the core was changed, i.e. relaxed to require the genes to be present in only 99% of the isolates. In **Figure 3** we displayed the effect of ten different increasingly stringent thresholds on the results of *in-silico* cgMLST. A 99% threshold was chosen to be displayed as the cgMST backbone structure in **Figure 1,** as it had the higher SDI value. This represented 4,342 loci. In the MST eight main cgMLST sequence types (cST) are present (each one containing more than 10 isolates) in our collection. We considered that two isolates belonged to the same cST if they differed in ≤ 5 loci.

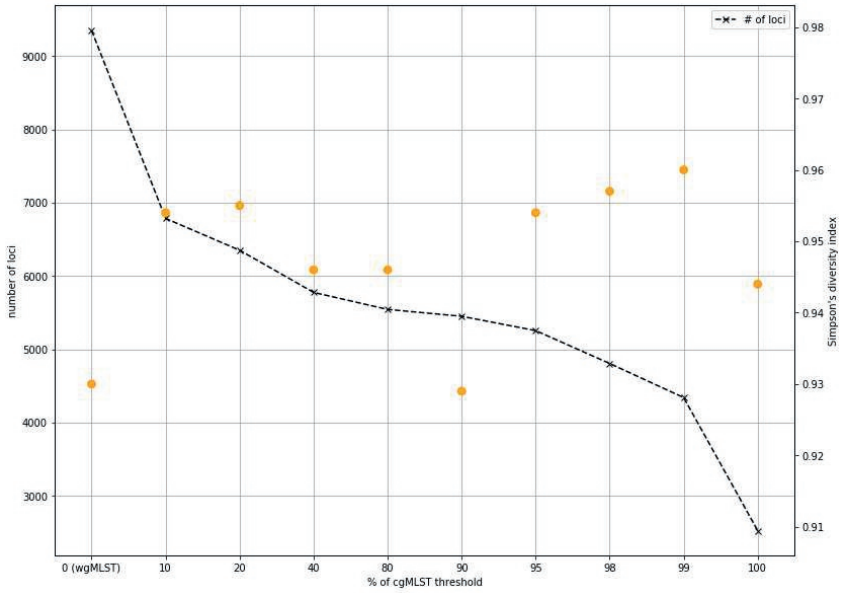

Figure 3. Correlation between the cgMLST threshold, the number of loci and the Simpson's diversity index (SDI). The cgMLST with a threshold of 0% is equivalent in number of loci and SDI value to the wgMLST scheme developed in BioNumerics®. As the cgMLST threshold is restricted, the number of loci in the scheme is reduced (black dashed line). The SDI values (orange dots) vary with each cgMLST threshold, and the threshold of 99% has the higher SDI value.

wgMLST

The wgMLST *P. aeruginosa* scheme available in the BioNumerics® software contains a total of 15,143 different loci. Using the A5-Miseq draft assemblies, the pipeline fetched 9,350 different loci throughout our collection, which represents 61,7% of the pipeline locus diversity. We considered that two isolates belonged to the same wgMLST sequence type (wST) if they

differed in ≤ 11 loci. In **Figure 1C** eight different wST were described, which are partly concordant with the cgSNPs grouping.

cgSNP

The cgSNP array is a concatenated string of 72,016-bp, which is about 1% of the maximum genome length recorded for *P. aeruginosa*. In this case, we considered that two isolates where indistinguishable if they differed in ≤ 4 SNPs. Again, similarly to the cg/wgMLST approaches, in **Figure 1D** we observed how the main cgMLST genotypes contain different core-SNP clusters (cSNP). The number of cSNP clusters was lower when using this approach than with the cg/wgMLST approaches.

For each of these techniques, we collected information regarding the number of loci or SNPs used, the number of types obtained, and the Simpson's diversity index. All this information is displayed in **Table 1**. The adjusted Wallace coefficient (AW) values for the *in-vitro* MLVA were over 0.98 when compared to the *in-silico* MLST ($AW_{\textit{in-vitro}\ \text{MLVA} \rightarrow \textit{in-silico}\ \text{MLST}}$ 0.988 [CI 95%, 0.966-1.000]) or the *in-silico* MLVA ($AW_{\textit{in-vitro}\ \text{MLVA} \rightarrow \textit{in-silico}\ \text{MLVA}}$ 0.983 [CI 95%, 0.961-1.000]), but below 0.6 when compared to the other WGS-typing methods. The *in-silico* MLST had the lowest AW values as compared to those for the other techniques. $AW_{\textit{in-silico}\ \text{MLST} \rightarrow \textit{in-vitro}\ \text{MLVA}}$ 0.462 (0.405-0.519) was its highest value and $AW_{\textit{in-silico}\ \text{MLST} \rightarrow \text{cgMLST 99\%}}$ 0.150 (0.114-0.186) its lowest, indicating that *in-silico* MLST cannot reliably predict clustering of the strains by the other, more discriminatory techniques. The wgMLST outperformed the cgSNP typing approach, as $AW_{\text{wgMLST} \rightarrow \text{cgSNP}}$ was 0.995 (0.990-1.000), but the difference was statistically non-significant. Vice versa, cgSNP poorly predicted clustering by cgMLST (0.552) or by wgMLST (0.564). Full results regarding AW values are displayed in **Table 2**.

DISCUSSION

The clinical microbiology field has four important axes which involve bacterial detection, bacterial identification, antibiotic susceptibility testing, and bacterial typing, the latter being the scope of this paper. For several years, typing techniques in most clinical microbiology laboratories were either based on the PCR-amplification of certain loci or on whole DNA fingerprinting using macro-restriction enzymes [29–31]. A recent review on typing methods by Chen, JW *et al*. [32] compared such typing tools specifically for *P. aeruginosa*. The authors thoroughly discussed the advantages and limitations of different molecular techniques highlighting the practical possibilities of genomics in the field of epidemiological investigations. In the years to come it is expected that NGS will gain even more terrain in the clinical microbiological field, despite its current limitations [33–35]. Recently PulseNet gave up PFGE as a gold standard and moved to WGS-typing standards [17], other institutions have followed this

initiative and have substitute "classic" molecular diagnostic typing techniques for WGS-based approaches [36]. This is a palpable reality in many reference microbiology laboratories around the world, but most routine clinical laboratories, and especially those in emerging or developing economies, are not there yet or are just transitioning towards this not-too-distant future practice. In the present paper we compared five typing approaches: classical 7-digit *in-silico* MLST, cgMLST, wgMLST and cgSNP analysis, and *in-vitro* MLVA. Since we already had obtained *in-vitro* MLVA data for the collection of isolates in this study, we could use this information to validate the *in-silico* MLVA approach, an option that is basically impossible for cgMLST, wgMLST and cgSNP analyses.

When comparing the performance of the five techniques, we considered two parameters, the SDI and the AW. Both have been used before for this purpose. While SDI is expressed as a single value, the AW scores are bi-directional representing the accuracy the clustering of strains by one technique to predict the clustering by another and *vice versa* (Method A → Method B and Method B → Method A); thus, the direction of the comparison matters. To use our data set as an example, when the *in-vitro* MLVA is compared to the *in-silico* MLST technique, the AW coefficient is 0.988. But when we analyze the techniques the other way around, the AW coefficient is only 0.462. This shows that *in-vitro* MLVA can predict the partitioning made by *in-silico* MLST very well (thus an AW coefficient close to 1), but at the same time *in-silico* MLST poorly predicts the partitioning made by *in-vitro* MLVA. Additionally, using the SDI value gives us another idea about the performance of the typing methodology. The higher this value, the sparser and diverse is the set of strains being analyzed. In the end, and using values obtained from AW and SDI, we can define which technique performs better.

We also validated an *in-vitro* 8-loci MLVA scheme and compared it to a homologous *in-silico* version, obtaining the loci from the WGS data. The discrepancies observed in **Figure 2** between the *in-vitro* and the *in-silico* approaches are highly marker-dependent and probably linked to limitations observed in WGS when dealing with repetitive DNA sequences [37]. In each locus of the MLVA scheme the number of copies which defines the VNTR number, will also determine the length of the fragment to detect. The larger the tandem repeat fragment, the higher will be the complexity of this region, and more problems will arise when short-read WGS-based assemblers try to reconstruct it. This might explain why most discrepancies occur in loci with a high tandem repeat-unit number. A recent study performing *in-silico* MLVA of the zoonotic pathogen *Brucella melitensis* described similar problems when the software used to perform an 8-loci *in-silico* MLVA could not retrieve at least one allele call from 43/103 samples [38]. In this study the authors reduced clustering errors by using a wider 16-loci MLVA panel.

Table 2. Values of the adjusted Wallace coefficient and its 95% confidence intervals.

	***in-vitro* MLVA**	***in-silico* MLVA**	***in-silico* MLST**	**cgMLST**	**wgMLST**	**cgSNP**
***in-vitro* MLVA**	**NA**	0.983 (0.961-1.000)	0.988 (0.966-1.000)	0.312 (0.250-0.373)	0.315 (0.252-0.377)	0.561 (0.503-0.620)
***in-silico* MLVA**	0.814 (0.747-0.881)	**NA**	1.000 (1.000-1.000)	0.266 (0.212-0.320)	0.268 (0.214-0.322)	0.475 (0.421-0.528)
***in-silico* MLST**	0.462 (0.405-0.519)	0.564 (0.505-0.624)	**NA**	0.150 (0.114-0.186)	0.151 (0.116-0.187)	0.269 (0.227-0.311)
cgMLST	0.965 (0.926-1.000)	0.993 (0.987-1.000)	0.995 (0.988-1.000)	**NA**	0.966 (0.927-1.000)	0.982 (0.958-1.000)
wgMLST	0.966 (0.929-1.000)	0.993 (0.987-1.000)	0.995 (0.988-1.000)	0.958 (0.923-0.993)	**NA**	0.995 (0.990-1.000)
cgSNP	0.976 (0.944-1.000)	0.996 (0.992-1.000)	1.000 (1.000-1.000)	0.552 (0.464-0.640)	0.564 (0.477-0.650)	**NA**

We observed that all WGS-typing techniques outperformed the *in-vitro* MLVA scheme, and among the WGS-typing techniques the cgMLST performed slightly better than the wgMLST or the cgSNP analysis, as it had higher diversity indices and AW values, but these differences were not statistically significant. The definition of core genome that we used refers to the genes present in our set of 242 strains, and not the minimum number of shared genes present in the species *P. aeruginosa* as a whole, which according to Valot and colleagues, is around 5,233 orthologues genes [39]. On the other hand, what we defined as the cgMLST could be a step forward to the definition of the species-specific *P. aeruginosa* core genome, as all loci obtained from the BioNumerics® wgMLST pipeline have been curated, and, therefore, could potentially serve as a standard reference. Our cgMLST approach has also some drawbacks, the more obvious being the lack of inter-laboratory reproducibility, as adding or removing samples effects the number of genes present in the cgMLST scheme.

Our results support the common consensus that WGS-based bacterial typing approaches have higher discriminatory power than other (DNA-based) techniques, while maintaining epidemiological cluster identification if cut-off values are well defined. This has been proven for several pathogens such as *Salmonella* typhimurium, *Listeria monocytogenes* or *Mycobacterium tuberculosis* [40–42]. In this study we have used WGS techniques that use either cgSNP-based or a gene-by-gene method. The specific characteristics of the software used in this paper have been described earlier in a review describing bioinformatic software for nosocomial outbreak analysis [19]. The SNP approach has two main variants depending on the use of a reference strain in the analysis or not. The latter is the preferred choice, since using a reference strain would require choosing a reference strain that is very closely related to the analytes in order to maximize the SNP identification and reduce mis-mapping events (i.e.: analyte reads mapping against an unrelated region of the reference genome). The quality of the input samples and filtering steps have also an impact in the results, regardless of the SNP approach chosen. There is a broad choice of software tools to perform all these tasks, some of them have also been reviewed earlier [43,44]. SNP-based analysis offers other additional advantages, such as tracking mutations related to AMR or phylotyping (defining evolutionary relationships). The other approach used in this paper is based on gene-by-gene detection and allele classification. This approach requires a curated and updated database containing a variable number of genes and its alleles (7-loci for the MLST schemes or thousands of loci for the core and whole genome MLST extensions). The pubmlst.org domain hosted by the Zoology department of Oxford University contains the MLST schemes for dozens of bacterial and eukaryote pathogens [45]. There are also public domain schemes for core and whole genome MLST, which are available for many (but certainly not all) pathogens [44]. Properly curated schemes allow a standardized nomenclature (i.e.: discussing the same gene,

properly annotated and referring to the same allele numbers) which facilitates data exchange and inter-laboratory reproducibility, both essential requirements in molecular epidemiology.

CONCLUSIONS

Before the full implementation of WGS as a diagnostic tool in the routine medical microbiology laboratory, important technical, scientific, and economical barriers need to be taken (i.e.: standardization of quality controls and protocols, AMR prediction for certain bugs, improved scalability, portability and traceability of analysis, and competitive prices per sequenced strain). In most medical microbiology laboratories, the transition from classical molecular typing to NGS-typing methods will not happen overnight. Therefore, alternative and accurate typing methods such as the MLVA scheme presented in this paper are likely to continue to play a key role in infection control strategies and the local epidemiological surveillance tools repertoire. MLVA constitutes an appropriate tool for laboratories that remain dependent on classical typing methodologies, but also for laboratories transitioning to NGS methods or those that remain faced with limited economic resources. It is a rapid approach with a high resolution, compatible with studying single- or even multi-center *P. aeruginosa* epidemiology.

On the other hand, for laboratories using WGS-typing methods, the wgMLST *P. aeruginosa* scheme would appear to be the best option, as it has high resolution and offers a curated loci database and a level of standardization which would allow global-scale inter-laboratory epidemiological studies, using the same software.

DECLARATIONS

Ethics and regulatory considerations

The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the research on 17th September 2012, No: 561/PT02.FK/ETIK/2012, (No: 757/UN2.F1/ETIK/X/2014). A Material Transfer Agreement (MTA) was reviewed and approved by the Director of National Institute Research and Development, Ministry of Health (No: LB.02.01/I.9.4/8500/2013). The study was registered at www.trialregister.nl (No: 5541).

Consent for publication

Informed consent was documented by the use of a written consent form approved by the Ethics Committee Faculty of Medicine Universitas Indonesia / Dr.Cipto Mangunkusumo General Hospital and signed and dated by the subjects/guardians and by the person who conducted the informed consent discussion and two witnesses. The signature confirmed the consent was based on information that had been understood.

Competing interests

The authors declare that they have no competing interests in relation to the content of the present study. ACP, DDC, and AvB are employees of bioMérieux, a company that develops and sells diagnostic tests in the field of infectious diseases.

Funding

Andreu Coello Pelegrin was funded by the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie Grant Agreement No 675412 (New Diagnostics for Infectious Diseases [ND4ID]).

Yulia Rosa Saharman is an awardee of the DIKTI-NESO Scholarship by The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, and Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands.

Authors' contributions

YRS, AK, HAV, and JAS conceived the study and participated in design of the study. YRS, RS, and DA participated in acquisition of data. ACP, CHWK, AvB, and JAS performed data analysis and interpreted the data. ACP, YRS, HAV, CHWK, DDC, JAS, HG and AvB drafted the article. All authors participated in critically revising the draft. All authors read and approved the final manuscript.

Acknowledgements

We are thankful to The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, Dean of Faculty of Medicine Universitas Indonesia, Board of Directors of Dr. Cipto Mangunkusumo National General Hospital Jakarta Indonesia, Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands and Critical Care Division, Department of Anesthesia and Intensive Care, Faculty of Medicine Universitas Indonesia / Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia.

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SUPPLEMENTAL MATERIALS

Table S1. Main quality parameters of the nine additional Carbapenem-susceptible *P. aeruginosa* strains sequenced.

Sample ID	Instrument	Assay	Average read length (nt)	# total reads	Assembly length (bp)	Assembly Coverage (X)	# Contigs	N50 (bp)	GC (%)	# N's per-100 kbp
BS3258	NextSeq	Nextera XT	150	8.187.352	6.670.366	184	113	129.965	66	6,4
BS3318	NextSeq	Nextera XT	150	6.499.576	6.372.765	153	71	268.426	66	3,0
BS3342	NextSeq	Nextera XT	150	4.869.186	6.280.171	116	38	425.981	67	2,1
BS3343	NextSeq	Nextera XT	150	3.189.762	6.435.138	74	64	355.372	66	5,6
BS3351	NextSeq	Nextera XT	150	3.834.608	6.580.949	87	45	515.408	66	5,2
BS3352	NextSeq	Nextera XT	150	3.704.630	6.445.545	86	30	516.959	66	5,1
BS3353	NextSeq	Nextera XT	150	3.137.492	6.446.086	73	43	432.105	66	5,9
BS3357	NextSeq	Nextera XT	150	5.057.624	6.402.421	118	39	602.168	66	3,6
BS3408	NextSeq	Nextera XT	150	4.884.196	6.882.698	106	54	342.677	66	3,7

Text S1. Construction of a whole genome MLST scheme for *P. aeruginosa*

A whole genome multilocus sequence typing (wgMLST) scheme was created by bioMérieux (Applied Maths NV) using 2,287 publically available *P. aeruginosa* genome datasets. First, a set of 400 reference genomes representing the known diversity within the species was selected. To this end, locus sequences are gathered from the type strain's genome (strain PAO1, RefSeq accession NC_002516.2), where each sequence annotated as gene coding region (CDS) is considered a locus. Using a BLAST approach and these type strain's loci, new alleles for each of the type strain's loci are detected in the other genomes. The genomes that offer the largest number of new alleles are added to the actual set of reference genomes that will be used to develop the wgMLST scheme. The addition of genomes will continue until saturation, i.e. until the addition of new genomes to the set of reference genomes does not result any longer in the addition of new alleles. This procedure is similar to determining the distance between the strains and adding strains that are most distant to the ones in the current list and makes sure that the creation set will contain the genomes that reflect the largest diversity of the species under consideration. From these 400 reference genomes, an initial set of loci was determined using the coding sequences (CDS). Within this set, loci that overlapped more than 75% or that yielded BLAST hits at the same position within one genome were omitted or merged until only mutually exclusive loci were retained while preserving maximal genome coverage. Mutually exclusive loci are defined as loci for which the reference alleles (typically one or two unique DNA sequences per loci) only yield blast hits at a threshold of 80% similarity to their own genomic location and not to reference alleles of another locus, such as paralogs or repetitive regions. In addition, loci that had a high ratio of invalid allele calls (e.g. because of the absence of a valid start/stop codon [ATG, CTG, TTG, GTG], the presence of an internal stop codon [TAG, TAA, TGA], or non-ACTG bases) and loci for which alleles were found containing large tandem repeat areas were removed. Lastly, multi-copy loci, i.e. repeated loci for which multiple allele calls were retrieved, were eliminated to achieve 90% of the genome datasets used for scheme validation had less than 10 repeated loci. The resulting scheme contained 15,136 loci (including the seven loci from the previously published MLST scheme (1)), which are accessible through a plugin in the BioNumerics® Software (bioMérieux).

To determine the allele number(s) corresponding to a unique allele sequence for each locus present in the genome of a strain, two different algorithms are available were employed: the assembly-free (AF) allele calling uses a k-mer approach starting from the raw sequence reads while the assembly-based (AB) allele calling performs a blastn search against assembled genomes with the reference alleles of each loci as query sequences. After each round of allele identification, all available data from the two algorithms (AF and AB) were combined into a single set of allele

assignments, called consensus calls. If both algorithms returned one or multiple allele calls for a given loci, the consensus is defined as the allele(s) that both analyses have in common. If there is no overlap, there will be no allele number assigned for this particular locus. If for a specific locus the allele call is only available for one algorithm, this allele call will be included. If multiple allele sequences were found for a consensus locus, only the lowest allele number is retained. Only those genes are assigned an allele number that have valid start/stop codons and do not exceed a defined maximum of ambiguous bases and N's.

Scheme validation

In a first step to validate the scheme, the 1,887 publically available genomes that were not used to create the scheme were analyzed using a BLAST approach and the freshly created scheme. From these results the number of loci retrieved based on the annotation of the genomes (CDSses) is compared to the number of loci retrieved by application of the scheme. The final coverage for each genome is calculated as the ratio of % of bases in loci over the % of bases in CDSses. The average obtained coverage was 96.6%. Additionally, less than 0.2% of the bases of the loci in the scheme were found in multiple loci.

In a second validation step, 24 collections of repeatedly sequenced strains as well as sequence reads sets from published work (2–4) were (re)analyzed with the wgMLST scheme in BioNumerics (v7.6.3). Identical allelic profiles were obtained for the technical replicates (i.e. same sequence read set analyzed multiple times), while for more than 90% of the collections with biological replicates (i.e. sequencing data obtained from different fresh cultures of the same strain) a difference of maximum 3 consensus allele calls were detected. For two collections of biological replicates between 7 and 10 differences were detected between the allelic profiles. However, these differences could be confirmed using a whole genome SNP approach. Results from the previously published work, which were obtained through a core genome or whole genome SNP approach, were confirmed by the wgMLST analysis with the *P. aeruginosa* scheme.

Text S2. Construction of the core genome MLST scheme for our *P. aeruginosa* isolates

The loci called from the wgMLST scheme were used to retrieve what we called the cgMLST. After running our samples through the wgMLST scheme, for each called locus by the scheme we calculated which percentage of our isolates had called an allele. Then we retrieved different sets of loci, using different percentage of "called loci" in our collection of isolates. i.e.: cgMLST 95%, means that from all loci called in the wgMLST scheme, we would retrieve those loci that are present in ≥ 95% of the isolates.

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CHAPTER 7

7

A multifaceted hand hygiene improvement program on the intensive care units of the National Referral Hospital of Indonesia in Jakarta

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ABSTRACT

***Background**:*

Hand hygiene (HH) is considered to be the single most effective measure in preventing healthcare-associated infections. However, HH compliance rates among nurses and doctors in hospitals are often very low. Few studies have addressed HH compliance in Indonesia, performed interventions to increase HH compliance, and none have had long-term follow-up. We, therefore, addressed this issue by performing long-term follow-up after a multifaceted intervention in the intensive care unit (ICU) setting.

***Methods**:*

This was an observational, prospective, before-and-after intervention study (May-September 2014, February-April 2017). We measured HH knowledge and HH compliance before (at baseline) and directly after a multifaceted improvement program (post-intervention) and performed a re-evaluation three years later. The multifaceted improvement program included education, feedback, reminders, interviews and the use of role models. The study involved nurses and physicians working in two ICUs of the Dr. Cipto Mangunkusumo Hospital in Jakarta.

***Results**:*

A total of 97 at baseline, and 72 at post-intervention HH knowledge questionnaires were completed. There was a statistically significant improvement in the mean overall HH knowledge score at post-intervention (from 14.3 to 20.8, $p<0.001$). There was no significant difference between the two ICUs. The overall HH compliance was 27% at baseline and significantly improved to 77% post-intervention ($p<0.001$). For all five HH moments, the compliance of nurses and physicians separately improved significantly from the baseline phase to the post-intervention phase ($p<0.0001$), except for 'moment 3' (after body fluid exposure), for which baseline rates were already high. Most of the compliance rates were significantly lower in both groups of healthcare workers upon follow-up three years later. Overall, the HH compliance of the nurses was significantly better than the physicians' compliance ($p=0.005$).

***Conclusions**:*

Our multifaceted improvement program, for nurses and physicians of the ICUs in the largest hospital of Indonesia, resulted in a significant improvement of the HH knowledge and HH compliance, but HH compliance levels waned over time after the intervention, indicating a need for continued monitoring and repeated interventions.

Trial registration:

The study was registered at www.trialregister.nl (No: 5541).

Candidate number: 23527, NTR number: NTR5541, Date registered NTR: 22-DECEMBER-2015

Keywords:

hand hygiene, multifaceted improvement program, compliance, Intensive Care Unit, Indonesia

INTRODUCTION

In both developed and resource-poor countries, healthcare-associated infections (HCAIs) have a large impact, especially those with multidrug-resistant (MDR) bacteria.[1, 2] One fourth of HCAIs involve patients in intensive care units (ICUs) and among them, the burden of infections by MDR microorganisms is the highest.[3, 4]

The World Health Organization (WHO) identified the risk of acquiring a HCAI as being universal. The importance of hands in the transmission of infectious agents has been demonstrated. HH is considered to be the single most effective measure not only reducing the spread of microorganisms, but also preventing HCAIs.[3] Despite this fact, studies in developed countries, especially with a mid-level sociodemographic profile, have reported very low compliance rates, including, India: 3-50.2% [5]. Vietnam: 43.6% [6], and other cities in Indonesia: Semarang (22.0% and 46.0%) [7], Malang (5.2%, 10.1% and 24.1%) [8]. In a recent systematic review of the global HH literature, Erasmus *et al*. found a median HH compliance rate of 40% with a range of 4 -100%.[9]

A recent study on potential determinants of HH compliance showed that, besides the perception of the healthcare workers (HCWs) that there is a lack of evidence that HH is effective in preventing HCAIs, a lack of positive role models and social norms may hinder compliance.[10]

Another study which took place on the island of Java, Indonesia, found that questionnaires in conjunction with site visits and interviews was a valuable strategy to identify trouble spots in the hospitals and to determine barriers of change that should be taken into account when planning interventions.[11]

The complexity of the process of behavioural change suggests that the application of multimodal, multifaceted strategies is necessary. Multifaceted strategies seem to result in a larger improvement of HH compliance (27-83%) as compared with using a single strategy (4-18%). [12] Education with written material, reminders, and continued feedback of performance can have an important effect on HH compliance.[12]

Using this information on HH improvement strategies, we developed a multifaceted improvement program to apply on the ICUs in the largest hospital of Indonesia, a country currently considered to have a mid-level sociodemographic index. This multifaceted improvement program was based on WHO tools and included education, feedback, reminders, interviews and the use of role models.[13] The aim was to improve the HH knowledge and HH compliance of the nurses and physicians working on the ICUs of the Dr. Cipto Mangunkusumo Hospital in Jakarta. The study was part of a larger study focusing on the reduction of transmission of MDR bacteria in the ICU.

METHODS

Study design

We performed an observational, prospective, before-and-after study in two ICUs of the largest hospital of Indonesia. Dr. Cipto Mangunkusumo Hospital is a 1200-bed university hospital located in Jakarta. At the time of this study up to 86% of patients did not have health insurance and had to pay for their hospital stay, medicines and laboratory tests. To improve HH knowledge and HH compliance among physicians and nurses we performed a multifaceted improvement program. This program was applied to the general ICU as well as to the ICU of the Emergency Department (ER-ICU), which is housed in a different part of the hospital but is managed by the same staff of Intensive Care Medicine.

The study consisted of four study phases (Table 1). In phase I, also referred to as baseline, which lasted from May 22nd – June 29th,2014, we measured HH compliance by means of anonymous observations after which we performed HH knowledge tests through questionnaires completed between June 30th - July 7th, 2014. Phase II was the intervention that lasted from July 10th – August 29th, 2014, and which consisted of the implementation of a multifaceted improvement program that included education, feedback, reminders, interviews and the use of role models. Phase III was the post-intervention period from September 2nd – 30th, 2014, during which period HH knowledge was again measured through the same questionnaires as in phase I (from 1st-4th of September, 2014) and in which HH compliance was again measured through unobtrusive observations. Long-term evaluation of HH compliance was evaluated in phase IV (which lasted from February 20th – April 10th, 2017) by using the same protocols.

Table 1. Study phases and activities performed

Study phase	Activity	Date
Phase I: baseline	Hand hygiene compliance observations: healthcare workers	22 May- 29 June 2014
	Hand hygiene knowledge questionnaire: nurses and physicians	30 June-7 July 2014
Phase II: intervention	Multifaceted hand hygiene improvement program*	10 July – 29 August 2014
Phase III: post-intervention	Hand hygiene compliance observations: healthcare workers	2- 30 September 2014
	Hand hygiene knowledge questionnaire: nurses and physicians	1-4 September 2014
Phase IV: long-term evaluation	Hand hygiene compliance observations: physicians and nurses	20 February – 10 April 2017

* Interventions are specified in Table 2.

Study setting and population

HCWs who participated in this study included all physicians on rounds in the ICUs, intensivists, all residents, nurses, students (medical students and nursing students), involved in patient care in these ICUs. HCWs were categorized into two categories, "physicians" and "nurses", for the sake of simplicity. At the individual level, the mix of participants differed somewhat in each phase.

In the general ICU, 43 nurses and 8 physicians were employed. This ICU has fifteen beds. In the ER-ICU, 34 nurses, 6 student nurses and 6 physicians were employed. The ER-ICU has six beds. Two nurses and one physician were involved in the HH improvement strategies and were, therefore, not subjected to observations, interviews and questionnaires. At the start of the study, each patient bed had one wall-fixed, alcohol-based liquid hand disinfectant dispenser with alcohol-based hand rub based on the WHO formula.[13] Per two patient beds there was one sink with a medicated soap dispenser (Cutisoft(@)), containing 4% chlorhexidine, and one wall-fixed paper towel dispenser. There was no possibility of improving these basic facilities owing to the financial circumstances.

Hand hygiene improvement strategies

Based on the study by M. Tromp *et al.*, we developed an improvement program which included education, feedback and reminders.[12] Education was given in the form of interactive lessons developed by the WHO, including formal lectures, practical demonstrations and written material.[13] In each lesson, we emphasized that alcohol-based hand rub is superior to traditional handwashing as it requires less time, acts faster, irritates skin less often, and proved to be contributing significantly to sustainable improvement in compliance, which was associated with decreased infection rates.[14] Only when hands are visibly dirty or are visibly soiled with blood or other body fluids, hand washing with either non-antimicrobial soap and water or an antimicrobial soap and water was indicated.[15]

Behavioural theories suggest that performance can by changed by feedback. In phase II and phase III personalized and non-personalized performance feedback was given to nurses and physicians of both wards. In several studies reminders were shown to have a sustainable effect on HH compliance. We gave reminders in the form of posters with handwashing messages placed on prominent sites in both ICUs.[16]

In addition, we conducted interviews to determine the importance of social influence. The seven group interviews took 40-60 minutes. Each group interview consisted of 5-10 participants, both nurses and physicians. The interviews were led by a moderator. At the start of the interview, it was emphasized that there were no good or bad answers. All group interviews were recorded with a voice recorder. To ensure that all topics of interest were discussed an interview guide was developed and used in each interview. All performed strategies are summarized in Table 2 and targeted the nurses and physicians of the general ICU as well as the ER-ICU.

Table 2. Hand hygiene improvement strategies used during the study

Improvement strategy	Date
Education	July 2014
Educational training	
Practical demonstration	
Written material	
Reminders	July 2014
Posters with hand hygiene reminders	
Interviews	August 2014
7 group interviews	
Performance feedback	July 2014-September 2014
Bar charts: hand hygiene compliance rates at baseline	
Individual feedback during observations	
Role models	September 2014
Assigned role models that instructed and stimulated their colleagues	

Hand hygiene knowledge questionnaire

Based on the WHO's 'Hand Hygiene knowledge questionnaire' a local questionnaire was developed (Supplemental data 1). The questionnaire was designed by the researchers and consisted of eight questions (including 17 sub-questions) about knowledge and five about attitude, perceived obstacles and self-reported behaviour. Two of the questions about perceived obstacles were developed by a Dutch clinical microbiologist. The questionnaire was translated into Indonesian after a pilot was tested by an Indonesian infection control nurse and physician. The questionnaire survey was carried out anonymously.

Participants completed the questionnaire during sessions at which the researchers were present to supervise. The ward was written on the form directly after a participant completed the questionnaire.

Hand hygiene compliance observation

The gold standard to monitor compliance of HH is direct observation.[17]

There were four observers who worked in parallel in phase I, two observers had received training about the correct method of HH at the Unit Infection Prevention of the Erasmus MC University Medical Center that is accredited to do so. In phase I, two local fellows of clinical microbiology were enlisted as additional observers, who were trained and supervised by the

principal investigator and the Erasmus MC trained observers. Prior to their scoring in the study these two fellows did a comparative trial run on observing compliance together with the trained observers from Erasmus MC and their results were compared using a kappa statistic to determine the interrater reliability. The two Erasmus MC trained observers scored HH compliance during phases I and III. The observers of the HH compliance in the phase IV, three years later, were local medical doctors recruited, trained and supervised in HH observations by one of the principal investigators (YRS).

Individual HCWs were observed during routine patient care by the observers with respect to potential HH opportunities available. The HCWs were not made aware that they were being observed. The nurses and physicians were unaware of the true reason for the presence of the observers at baseline, since we mentioned a participation to a different study as a reason for having observers on the wards. In addition, to avoid a Hawthorne effect we elected, when designing the study, not to include the week in which questionnaires on hand hygiene were completed as part of the baseline observation period.

An observation list was developed based on the five moments for HH, and based on the WHO tools. The observations were carried out several times a week during differing time slots, but not during the weekend. Every episode of observation lasted about 30 to 60 minutes, but sometimes longer, also to reduce the Hawthorne effect.

The observation list contained 5 indications for HH: (1) before touching a patient, (2) before a clean/aseptic procedure, (3) after body fluid exposure risk, (4) after touching a patient (regardless of the use of sterile or nonsterile gloves), (5) after touching patient's surroundings.

HH compliance was defined as hand disinfection using alcohol-based hand rub or washing hands with soap and water following one of the above-mentioned indications. The applied HH indication(s) and the performed HH action were marked on the observation list by the observers.[12] The outcomes of these observations were presented as percentages of compliance representing the fraction of the number of times when hand hygiene should have taken place correctly, and the number of times it had actually taken place correctly. The researchers, who were also the observers, recorded possible opportunities for HH over a period, which included observations at baseline (phase I), observations in the post-intervention phase (phase III), and long-term evaluation (observation by RK and M) (Phase IV), on both wards.

The targeted minimum number of observations for the baseline and post-intervention phases of the study was 1,100 moments, as derived from a recent systematic review of previous HH compliance studies [9]. The WHO [13] points out that low numbers of observation are associated with wide ranges of the confidence intervals. When designing the study, we, therefore, elected to have at least 1,100 observations in each of the pre- and post-intervention phases of the study to keep the confidence intervals around the observed, overall compliance rates narrow.

Data analysis

For analysis of the questions regarding knowledge, correct answers were analysed as 'correct'; incorrect answers, missing values (including 'no answer' and 'do not know') were all categorized as 'incorrect'. The results of HH knowledge questionnaires were analysed in two ways: a) at the level of the individual HCW, i.e. number of correct answers per person, and b) at the level of individual questions asked, i.e. the percentage of correct answers given per question. For the first analysis each question was given the same value of one, such that each HCW received a score ranging from 0 - 25. To compare the scores of individual HCWs before and after the intervention (phase I and III, respectively), the scores were graphically plotted, and mean and median scores were calculated and compared using the Mann Whitney test for independent samples. Because no data were stored on the identity of each HCW per phase, the scores in different phases could not be linked to each other, and a paired statistical analysis was thus not possible.

The performance of each of the 25 questions was analysed by scoring the percentage of participants providing the correct answer, thus these question-level scores ranged from 0 to 100%. The effect of the intervention on the percentage correct answers of each question was analysed using Fisher's exact testing (two-tailed), and Mann Whitney testing was used to compare the medians across all 25 questions before and after the intervention.

For the questions about attitude, perceived obstacles and self-reported behaviour, the answers were used for the development of the improvement program. To determine the effects of the improvement strategy on compliance over time, we used linear mixed models for the repeated measurements of the compliance rates. The independent variables in this model were type of HCW (nurse or physician), phase (phase I, III, or IV), moment (a categorical variable for the 5 indications), and ward (general ICU or ER-ICU). All independent variables were coded as categorical variables. Two-way interaction effects between type of HCW, phase and moment were included in the model. Because the HCWs were not identified during data collection, the responses could not be linked between phases at the individual level. Instead we estimated the model for aggregated data, i.e. the outcome consisted of the average compliance rate (expressed as a percentage) for each combination of the variables type of HCW, phase, moment, and ward. This aggregation approach uses the fact that we can link outcomes over time at the level of a ward and allowed us to account for correlations between repeated measurements. The correlations were modelled by including a random intercept for each combination of type of HCW, ward, and moment in the model. We accounted for the substantial variation in the number of observations between different phases and moments by modelling the variance of the compliance rate as a function of the binomial variance (i.e. the observed compliance rate times (1-observed compliance rate) divided by the number of observations). The results of the linear mixed model analysis were summarized using the estimated marginal means, i.e. the predicted compliance rates adjusted for the effects of covariates, and presented graphically.

These estimated marginal means were compared between the phases for each combination of moment and type of HCW. The comparisons were adjusted for the effects of multiple testing using Tukey's method.

Concordance between the observers was analysed using a kappa statistic. A kappa value of >0.60 was considered good. For the linear mixed model, we used 'R', version 3.5.2 with packages nlme and emmeans. For other analyses, we used SPSS version 22.0 (SPSS, Inc, Chicago, IL.). In all analyses, a two-sided p-value <0.01 was considered statistically significant.

RESULTS

Interviews

Prevention of cross-infections was named by the participants as the main advantage of HH. Dryness and soreness of hands after performing HH were the main disadvantages brought up by the participants.

A lack of social control with regard to HH compliance was mentioned by nurses as well as physicians. Nurses had no difficulties in approaching other HCWs, even physicians, about their HH behaviour. However, physicians reported to have difficulties in approaching for example senior staff members, because of 'the culture' in the hospital. In addition, they mentioned that noncompliance among physicians could arise from lack of strong evidence supporting the effectiveness of HH for prevention of HCAIs. Participants, nurses in particularly, mentioned the need for positive role models. The presence of negative role models, nurses or physicians noncompliant with HH, was reported as reason for their own noncompliance.

Furthermore, participants noted that strategically placed reminders (posters, labels with messages) would help increase and maintain compliance with HH. Also, participants advised to regularly change the reminders into different ones in order to be sure that the reminders remain effective.

Hand hygiene knowledge

At baseline we measured the HH knowledge of 43 nurses and 8 physicians of the general ICU. At the ER-ICU 34 nurses, 6 physicians and 6 nurse students participated. The overall score of correctly answered HH knowledge questions for all HCWs and both wards combined ranged from 1 to 22, with a mean overall score of 14.3 (median 15; interquartile range (IQR) 13-16). Post-intervention we measured the HH knowledge of 32 nurses and 5 physicians of the general ICU. At the ER-ICU these were 31 nurses and 4 physicians. Of this group, the mean overall score of correctly answered knowledge questions for all HCWs and both wards combined was 20.8 (median 22; IQR 18-23), with a range from 13 to 25 (Figure 1). Compared with baseline, there was a significant improvement in the overall mean HH knowledge score post-intervention (p<0.001). There was no

significant difference observed in the overall mean score between the general ICU and the ER-ICU (p=0.692 by Mann-Whitney test, data not shown).

The multifaceted intervention resulted in significant improvement in HCW knowledge (median percent correct answer across all questions pre-intervention, 71% versus post-intervention phase, 89%; p=0.002). Supplementary Table 1 displays the results for the 25 individual HH knowledge questions separately, at baseline as well as post-intervention. For 15 questions, significantly more correct answers were given in the post-intervention phase compared to baseline. Interestingly, the question (5c) with the 2nd lowest number of correct answers in the pre-intervention phase (14.4%) significantly improved its score but had the lowest score in the post-intervention phase (52.8%); this question addressed the effectiveness of hand rubbing compared to handwashing. Thus, even after education and hand-written evidence almost half of the respondents remained convinced that handwashing with water and soap is more effective than hand rubbing with alcohol. In contrast, the rate of correct answers to question 2 improved from a very low 4.0% to 75.0%, this question dealt with the most prevalence source of pathogens causing nosocomial infections.

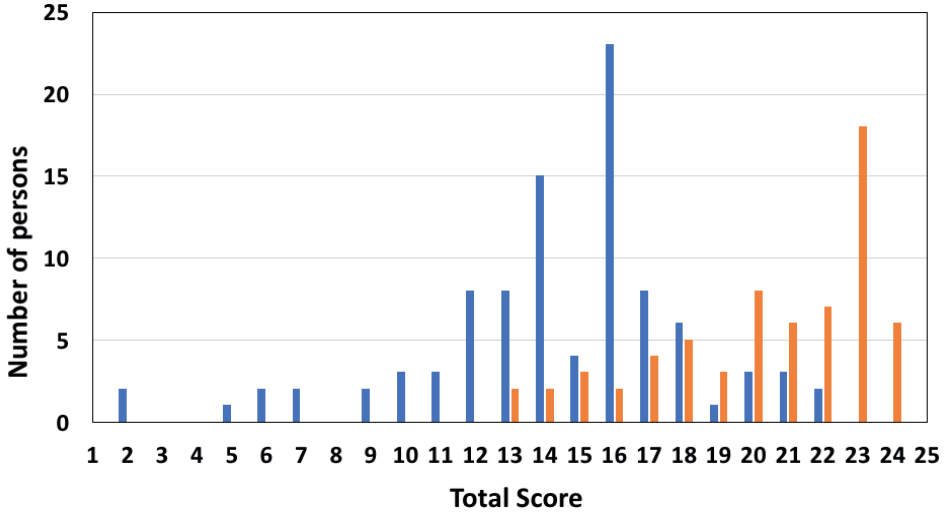

Figure 1. Effect of an educational intervention on hand hygiene knowledge of healthcare workers.
Legend: Bars indicate the number of persons achieving indicated overall score derived from questionnaires taken during the baseline (blue bar) and post-intervention (orange bar) phases of the study.

Hand hygiene compliance

In the general ICU and the ER-ICU combined, a total of 7187 HH opportunities was observed (Table 3). The observations between researchers and the clinical microbiology fellows were concordant (kappa 0.72) when tested in a pilot run in the general ICU.

The most frequently observed indications for HH were 'after touching patient's surroundings' 3126/7187 (44%) and 'after touching a patient' (24%) (Table 3).

Table 3. Number of opportunities for hand hygiene observed at baseline, in the post-intervention phase, and at long-term follow-up.

	Baseline	Post-intervention	Long-term evaluation	Total
Before touching a patient	386	218	705	1309
Before a clean/aseptic procedure	212	171	241	624
After body fluid exposure risk	108	59	267	434
After touching a patient	692	299	703	1694
After touching patient's surroundings	920	642	1564	3126
Total	**2318**	**1389**	**3480**	**7187**

The number of HH opportunities at baseline, both wards combined, was 2318 with an overall compliance rate of 27%. In the post-intervention phase, the number of HH opportunities was 1389 with an overall compliance of 77%. Thus, the overall HH compliance improved significantly from baseline phase I to the post-intervention phase III, but had regressed to the baseline level again when evaluated at long-term follow-up (33% in phase IV, Figure 2, Table 4). For all moments, the HH compliance of nurses and physicians separately improved significantly from phase I to phase III ($p<0.0001$), except for moment 3 (Figure 3, Table 4). Over the whole observation period, however, the HH compliance rates were higher among the nurses than among the physicians (44% versus 27%, $p=0.005$ in linear mixed model). There was no significant difference in compliance rate found between the two wards ($p=0.463$, data not shown).

We also analysed the sustainable effect of this intervention program by a long-term follow-up evaluation (phase IV), most of the compliance rates were significantly lower in both groups of HCWs (phase III versus phase IV), except for moment 3 (after body fluid exposure risk), for which both physicians and nurses had high compliance rates at baseline and remained so in phase III and IV, albeit that these values were statistically not significantly higher.

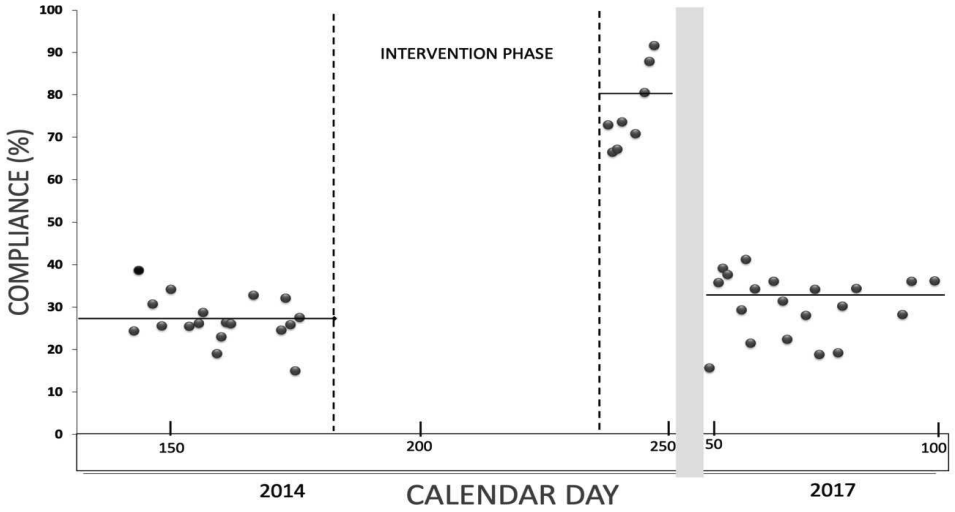

Figure 2. Time series of compliance rates per observation day.

Legend: Dots indicate the compliance rates per observation day. The observations used to calculate the compliance rates per day were conducted in the baseline phase and the post-intervention phase in 2014, and in the long-term evaluation phase in 2017. The horizontal lines represent the average of compliance rate in each phase.

DISCUSSION

This was an observational, prospective, before-and-after intervention study. The overall HH compliance rates at baseline phase were low in both ICUs (27%). The HH compliance level observed before intervention was similar to those reported from other countries that have a mid-level sociodemographic index [18] [5] [19] [20] [6], and other cities in Indonesia[7] [8].

Our study showed that overall a significant increase in the HH compliance of HCWs could be achieved using a multifaceted improvement program (compliance in post-intervention phase: 77%). In line with the report by Tromp *et al.* [12], we developed a multifaceted improvement program. Since this is a multifaceted approach, it is not possible to determine the contribution of each component to the observed improvement.

Duerink *et al*. introduced a multifaceted intervention study to improve compliance in a tertiary care hospital in the city of Semarang, Indonesia, and found this strategy increased HH compliance from 46.0% to 77.0% in the internal medicine ward and from 22.0% to 62.0% in the paediatric ward.[7] Santosaningsih *et al*. also performed interventions in a similar hospital in Malang, and improved HH compliance significantly in paediatric (24.0% to 44.0%) and internal medicine wards (5.0% to 19.0%), but also in the control ward, obstetrics-gynaecology, where no

Table 4. Hand hygiene compliance rates by nurses and physicians observed for each of the 5 HH moments at baseline, post-intervention and at long-term evaluation.

Moment, by healthcare worker	Compliance % (correct/total number)					
	Baseline (Phase I)	Post Intervention (Phase III)	Long-term evaluation (Phase IV)	p (I-III)*	p (I-IV)*	p (III-IV)*
1. Before touching a patient						
Nurses	21 (58/276)	88 (115/131)	31 (174/569)	**<0.0001**	0.0657	**<0.0001**
Physicians	13 (14/110)	66 (57/87)	18 (24/136)	**<0.0001**	0.7147	**<0.0001**
2. Before a clean/aseptic procedure						
Nurses	17 (33/194)	75 (118/157)	37 (80/215)	**<0.0001**	**0.0032**	**<0.0001**
Physicians	39 (7/18)	50 (7/14)	54 (14/26)	**<0.0001**	0.0775	**0.0037**
3. After body fluid exposure risk						
Nurses	69 (64/93)	91 (49/54)	85 (204/239)	0.0383	0.1032	0.5811
Physicians	80 (12/15)	80 (4/5)	75 (21/28)	0.8608	0.5276	0.8958
4. After touching a patient						
Nurses	49 (257/529)	93 (181/194)	70 (390/560)	**<0.0001**	**0.0003**	**<0.0001**
Physicians	39 (63/163)	72 (76/105)	43 (61/143)	**<0.0001**	0.0345	**0.0036**
5. After touching patient's surroundings						
Nurses	15 (95/616)	80 (370/463)	15 (152/1043)	**<0.0001**	0.9007	**<0.0001**
Physicians	6 (18/304)	54 (96/179)	3 (18/521)	**<0.0001**	0.2311	**<0.0001**

* Based on the linear mixed model analysis. Significant changes in compliance are in bold character

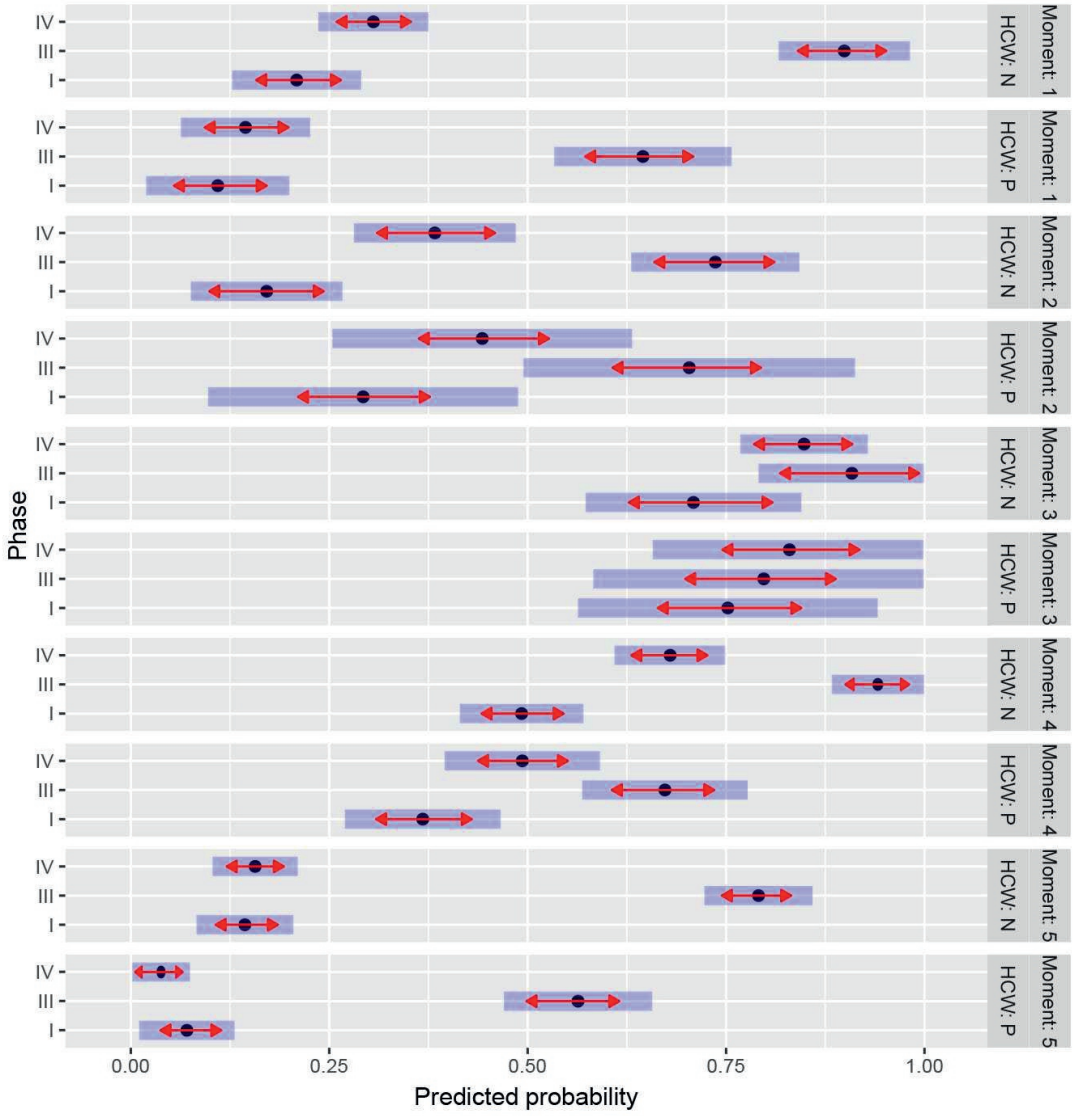

Figure 3. Predicted compliance rates by WHO moment of hand hygiene and by healthcare worker in the baseline phase (I) and post-intervention phases (III and IV).

Legend: HCW, healthcare worker**;** HCW: N: nurse; HCW: P: physician.

Moment 1 is before touching a patient. Moment 2 is before a clean/aseptic procedure. Moment 3 is after body fluid exposure risk. Moment 4 is after touching a patient. Moment 5 is after touching patient's surroundings. Black dots indicate the estimated marginal means of compliance rate. The red arrows give information on the significance of the difference between phases. Overlapping red arrows within a block (*i.e.* a block of phase I, III, and IV) means a nonsignificant difference between phases, nonoverlapping arrows imply a significant difference. The arrows are adjusted for multiple testing using Tukey's method. The blue bars are 95% confidence intervals, not adjusted for multiple testing.

no intervention had been performed (10.0% to 21.0%).[8] They concluded that role model training had the most impact.[8]

Our strategy was highly effective for the nurses as well as the physicians. Similar to previous studies [21], the overall level of HH compliance in our study was significantly higher among nurses (44%) than among physicians (27%). In the setting of our study, we noticed a clear difference in activities of nurses and physicians. Nurses play an important role in the daily care of the patients. Physicians have much fewer patient contacts. Therefore, the nurses have far more HH opportunities in their daily routine. This could explain why the nurses had a more pro-active attitude towards the improvement program with a higher HH compliance as result. This difference in HH compliance between nurses and physicians may also be due to the fact that not all physicians were convinced about the effectiveness of HH. Physicians mentioned that their noncompliance was associated with a perceived lack of evidence that hand hygiene is effective in the prevention of hospital-acquired infection, which could be an explanation for the inverse correlation found between the level of education and the rate of handwashing compliance. Better information about the available evidence might well promote better compliance.[16, 21]

In the analysis of the compliance rates per HH moment, there is a notable difference between 'moment 3' and the other four HH moments. The HH compliance rates for 'moment 3' at baseline (physicians: 80%, nurse: 69%), were already very high, compared to the HH compliance rates of the other HH moments. Moment 3 is defined as a HH indication after body fluid exposure risk. Other studies have shown that the HH behaviour of healthcare workers appears to be motivated by self-protection and a desire to clean oneself after a task that is perceived to be dirty, rather than protect their patients by proper HH before approaching the patients. This may explain the high HH compliance rate at baseline in our study.[21]

HH knowledge was indeed low at baseline, but showed a large increase in the post-intervention phase. Therefore, we hypothesize that education was an important component of the intervention.

Dryness and soreness of hands after performing HH were the main obstacles reported by participants of the interviews and in the HH knowledge questionnaire before the multifaceted intervention. The medicated soap and the hand alcohol suspensions did not contain moisturizers and emollients. Hand washing with 4% chlorhexidine medicated soap dries out the skin and results in pre-irritated skin. Applying alcohol-based hand rub to pre-irritated skin may cause a burning sensation. Because of the burning sensation the HCWs tend to reduce the use of alcohol-based hand rub and prefer hand washing with medicated soap, which is the underlying reason for the burning sensation. However, this fact was not stressed during the educational part of the intervention. Due to financial constraints, additional moisturizing hand creams were not available. Replacement of the medicated soap to plain mild soap would have been more in line with the WHO strategy.

The long-term effect of our multifaceted improvement program was assessed after three years. This follow-up showed that the intervention by and large did not have a sustainable effect on HH compliance, except 'moment 3' (after body fluid exposure risk) in both groups of HCWs. In Khatib *et al.*[22] the use of reminders showed a modest positive effect on HH compliance rates, but they were able to maintain higher compliance rates for a longer period of time. Other studies also showed that reminders have a modest sustaining effect on HH compliance rates.[16, 23] With the use of reminders and positive role models in our multifaceted improvement program, we strived to have a long-term effect on HH compliance rates.

Human behaviour is a complex process determined among others by knowledge about and attitude towards the behaviour, perceived social standards and self-efficacy.[11] Behavioural change is often viewed as a difficult process, also in the hospital. HCWs continue to fail in adherence to the guidelines for HH which hampers the reduction of HCAIs.[4] In several studies, the effectiveness of different HH improvement strategies have been described.[17] Erasmus *et al.* found that personal beliefs about the efficacy of HH and examples and norms provided by senior hospital staff are of major importance for HH compliance. They further reported that HH is most often performed after tasks that they perceive to be dirty, and personal protection appeared to be more important for compliance than patient safety. Physicians mentioned that their noncompliance arose from their belief that the evidence supporting the effectiveness of HH for prevention of HCAIs is not strong.[21]

Some possible limitations of our study must be considered. For measuring the HH compliance, we used direct observations using the standard observation form as defined by the WHO.[3] Direct observations have limitations; they are time-consuming, manpower intensive and continuous monitoring is currently not feasible in this setting. The information provided probably represents a low percentage of all HH opportunities. By mentioning our participation to a different study as reason for the presence of the observers, the nurses and physicians were unaware of the true reason for the observations at baseline. Despite of our long periods of observations, observation bias and the Hawthorne effect cannot be fully excluded. Also, the effectiveness of HH on the prevention of HCAIs depends on HH technique in addition to HH compliance.[24] HH technique training was a part of our program, however, this was not evaluated. A full system change was not achieved by the intervention. State of the art is to increase the use of alcohol-based hand rub and decrease hand washing. 4% Chlorhexidine medicated soap should have been removed from all wards. A mild liquid soap should have been provided instead of medicated soap. The observed decrease in HH compliance rates after the initial post-intervention phase could be, at least in part, the result of an incomplete system change.

CONCLUSIONS

In conclusion, our multifaceted improvement program, for nurses and physicians of the ICUs in the largest hospital of Indonesia, resulted in a highly significant improvement in the HH knowledge and HH compliance, but maintaining high levels of HH compliance requires continuous monitoring and regular interventions.

DECLARATIONS

Ethics approval and consent to participate

- The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the research on 17th September 2012, No: 561/PT02.FK/ETIK/2012, No: 757/UN2.F1/ETIK/X/2014. The study is registered at the Dutch National Trial Register (No: 5541).
- Consent to participate: Not applicable (does not report on or involve the use of any animal or human data or tissue).

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

YRS is an awardee of the DIKTI-NESO Scholarship by The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, and Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands.

All authors report no conflict of interest relevant to this article.

Funding

This work was supported by 'The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia' and 'Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands'.

Authors' contributions

YRS, DAF, SEA, AK, HAV, and JAS conceived the study and participated in design of the study.

YRS, DAF, SEA, RS, and DA participated in acquisition of data.

YRS, DAF, JvR, HAV, and JAS performed data analysis and interpreted the data.

YRS, DAF, SEA, JvR, HAV, and JAS drafted the article.

All authors participated in critically revising the draft.

All authors read and approved the final manuscript.

Acknowledgements

We are thankful to The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, Dean of Faculty of Medicine Universitas Indonesia, Board of Directors of Dr. Cipto Mangunkusumo National General Hospital Jakarta Indonesia, Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands and Critical Care Division, Department of Anesthesia and Intensive Care, Faculty of Medicine Universitas Indonesia / Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia, nurses and physicians of the adult ICU and ER-ICU of Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia for completing and returning the questionnaires and for giving us the opportunity to carry out the observations, Ns Sondang Sihite (Infection Prevention Control Nurse adult ICU), Ns Eliyani (Infection Prevention Control Nurse ER-ICU), Ratna Kencana MD and Marina MD as an observer on phase IV, Clinical Microbiology fellow of Department of Clinical Microbiology Faculty of Medicine Universitas Indonesia / Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia.

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SUPPLEMENTAL MATERIALS

Supplementary Table 1. Survey results regarding hand hygiene knowledge questions.

	Baseline (%) correct answer	Post-intervention (%) correct answer	**P**-value (2 sided)
Overall median	15 (71)	22(89)	**<0.0001**
Question 1	79 (81.4)	50 (69.4)	0.099
Question 2	4 (4.1)	54 (75.0)	<0.0001
Question 3a	92 (94.8)	72 (100)	0.072
Question 3b	17 (17.5)	50 (69.4)	<0.0001
Question 3c	83 (85.6)	69 (95.8)	0.037
Question 3d	17 (17.5)	49 (68.1)	<0.0001
Question 4a	23 (23.7)	55 (76.4)	<0.0001
Question 4b	86 (88.7)	67 (93.1)	0.430
Question 4c	20 (20.6)	56 (77.8)	<0.0001
Question 4d	83 (85.7)	66 (91.7)	0.336
Question 5a	74 (76.3)	69 (95.8)	<0.0001
Question 5b	15 (15.5)	50 (69.4)	<0.0001
Question 5c	14 (14.4)	38 (52.8)	<0.0001
Question 5d	26 (26.8)	45 (62.5)	<0.0001
Question 6	69 (71.1)	69 (95.8)	<0.0001
Question 7a	80 (82.5)	70 (97.2)	0.003
Question 7b	69 (71.1)	69 (95.8)	<0.0001
Question 7c	90 (92.8)	72 (100.0)	0.021
Question 7d	29 (29.9)	48 (66.7)	<0.0001
Question 7e	45 (46.4)	64 (88.9)	<0.0001
Question 7f	89 (91.8)	71 (98.6)	0.080
Question 8a	84 (86.6)	70 (97.2)	0.026
Question 8b	61 (62.9)	43 (59.7)	0.750
Question 8c	78 (80.4)	65 (90.3)	0.088
Question 8d	58 (59.8)	63 (87.5)	<0.0001

Differences between baseline and post-intervention data were assessed using Fisher's exact test and Mann Whitney test. The written text of each question can be retrieved from the supplementary dataset.

CHAPTER 8

8

Multimodal Intervention to Reduce Acquisition of Carbapenem-Non-Susceptible Gram- Negative Bacteria in Intensive Care Units in the National Referral Hospital of Indonesia: An Interrupted Time Series Study

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Submitted

ABSTRACT

Objective:

Intensive care units (ICUs) are high-risk areas for transmission of Carbapenem-non-susceptible Gram-negative bacilli, especially in resource-limited settings. We evaluated the effect of a multimodal infection control intervention on the acquisition of Carbapenem-non-susceptible *Acinetobacter baumannii-calcoaceticus* complex*, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa.*

Design:

A quasi-experimental before-and-after design study that consisted of a pre-intervention phase 1 (2013-2014), an intervention phase 2 (2014-2015) and a post-intervention phase 3 (2015-2016).

Setting:

Two ICUs of the Indonesian national referral centre in Jakarta, Indonesia.

Patients:

All consecutive patients > 18 years, admitted for >48 hours.

Intervention:

Introduction of a multimodal infection prevention bundle.

Measurements:

The number of acquisitions/100 days at risk was determined and analyzed with a multilevel Poisson segmented regression model.

Results:

387 patients were observed in phase 1and 361 in phase 3. There was a significant step change, from phase 1 to phase 3 in the rate of acquisition of carbapenem-non-susceptible strains, the incidence rate ratio (IRR) was 0.374 (CI_{99}: 0.178-0.785) for phase 3 compared to phase 1. This significant decrease was mainly due to reduced acquisitions of resistant *A. baumannii* (IRR 0.438, CI_{99} 0.181-1.061) and *K. pneumoniae* (IRR 0.346, CI_{99} 0.091-1.31). Within each of the two phases there was no major downward or upward trend observed in the rate of acquisition of resistant strains for any of the three species separately nor for the three species taken together, although the risk of acquisition was increasing slightly in phase 1.

Conclusion:

A multimodal intervention aiming to prevent acquisition of resistant strains of important ICU pathogens is feasible and may be quite effective in ICUs in resource-limited settings. To control multidrug resistant *P. aeruginosa*, however, additional strategies should be explored.

Keywords:

acquition rate, carbapenem-non-susceptible**,** *Acinetobacter baumannii-calcoaceticus* complex*, Klebsiella pneumoniae, Pseudomonas aeruginosa,* ICU, multimodal interventions, Jakarta, Indonesia

INTRODUCTION

Multidrug-resistant (MDR) microbial pathogens have emerged worldwide as major concerns both in and outside of the hospital environment. Carbapenem-non-susceptible Gram-negative bacilli of the species *Klebsiella pneumoniae, Acinetobacter baumannii-calcoaceticus* complex, and *Pseudomonas aeruginosa* are amongst the most dreaded emerging threats. These pathogens have been highlighted as critical pathogens by the World Health Organization (WHO) prioritization of pathogens to guide discovery of new antibiotics.(1, 2) MDR pathogens pose tremendous challenges to healthcare systems, including challenges related to diagnosis, treatment, and containment of infections caused by them.(3) These challenges are amplified in the intensive care unit (ICU) environment, where pressures for the selection and emergence of resistance and risks of transmission of MDR pathogens are highest, and where the threat of potentially multiple drug resistance is a major driver of the prescription of empiric broad spectrum antimicrobial regimens.(4) Effective and targeted infection prevention and control (IPC) interventions, including contact precautions, environmental cleaning, and a hand hygiene improvement strategy are deemed essential to control the spread of carbapenem-non-susceptible Gram-negative bacilli in such settings. In general, multimodal interventions are more effective than a single mode of intervention.(5, 6) However, in lower-middle income countries (LMICs), including Indonesia, such multimodal interventions need not only to be effective but also inexpensive and relatively simple to apply. Such multimodal interventions have been little studied in these settings.

Therefore, we designed and evaluated the effectiveness of a low-cost multimodal infection control bundle at two ICUs in Jakarta, Indonesia, on the acquisition of carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex*, K. pneumoniae,* and *P. aeruginosa*.

METHODS

Study setting

We performed a study in two ICUs (adult ICU and Emergency Room (ER)-ICU) of the National Referral Hospital of Indonesia with 1,200 beds, located in Jakarta. These ICUs were multibed open wards in which multidisciplinary teams provide patient care under the final supervision of intensivists (for physical layout see supplementary material). These ICUs together admitted approximately 1,400 patients per year, both surgical and medical patients, and it also functioned as a post-anesthesia care unit after major surgery. There were ~50 healthcare workers (HCWs) proving care for patients in each unit, including three intensivists, anesthesiologist residents, nurses and other HCWs. Nurses did not rotate between the two ICUs, but the intensivists did. More details on patient-to-nurse ratios are described elsewhere (7, 8). Cleaning of the ICU environment was outsourced and initially limited to mopping the floors twice daily with a detergent solution without

systematically attending the doors, walls, wall fixtures, sinks, beds and instruments. Solutions containing quaternary ammonium salts where occasionally used for disinfection purposes.

Study design

The study used a quasi-experimental before-and-after design. All consecutive patients (≥18 years old) admitted to one of the two ICUs and hospitalized for more than 48 h were eligible for enrollment in this study. After a baseline phase, we introduced the interventions in a staggered fashion, as shown in figure 1. Thus, the study consisted of three phases: phase 1, a baseline observation period (1 April 2013–30 October 2013 and 18 April 2014 - 9 July 2014), followed by two intervention phases, phase 2 in which control measures were introduced (10 July 2014-31 January 2015) and phase 3 in which all measures were in effect (2 February 2015- 8 January 2016).

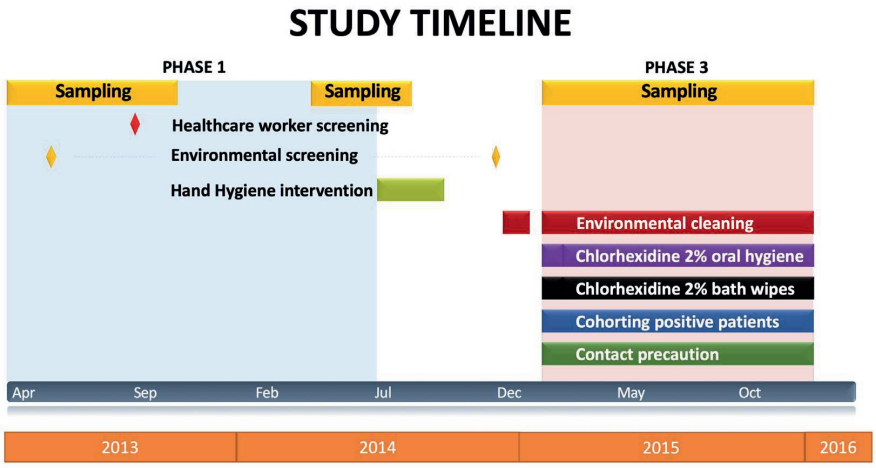

Figure 1. Study Timeline.

Phase 1: baseline surveillance.

Screening swabs were taken from throat and rectum or stools from all included patients on the day of admission, at the time of discharge from the ICU, and weekly if patients were admitted for seven days or more. Clinical samples were collected on indication from patients under aseptic precautions from the lower respiratory tract, blood, urine, tissue, or wound.

Environmental samples were taken once from various sites, and all HCWs working in one of the ICUs were sampled once over the course of one month (September 2013, Figure 1). All screening swabs and clinical samples were analysed according to a previously published protocol.(7-9)

Phase 2: infection control interventions.

After phase 1, we introduced a multimodal bundle of IPC interventions that initially consisted of the following measures:

- A multifaceted hand hygiene improvement program. We based the hand hygiene programme on the WHO's Five Moments for Hand Hygiene guidelines and tools. The hand hygiene improvement program included education with pre- and post-questionnaires testing of knowledge and attitudes, performance feedback and reminders, interviews, and role models as described before.(10)
- A single round of an environmental disinfection campaign involving the whole environment of both ICUs using 1:100 sodium hypochlorite solution. This disinfectant solution was applied to walls, floors, doors, beds (mattresses and bed rails), sinks, overbed tables, infusion and suction pumps and stands, monitors and ventilators including connecting lines, surrounding counter tops including the adjacent cleaning service room. In addition, all curtains between beds were exchanged for clean ones.
- Enforced antibiotic stewardship (including daily evaluation of all antibiotic prescriptions on weekdays)

During most of this phase the screening of patients was temporarily interrupted (Figure 1), and no additional screening of the HCWs was performed, but the environment was sampled once more just before the intensive cleaning campaign started.

Phase 3: additional IPC measures taken

In this phase, routine environmental disinfection was performed with 1:100 sodium hypochlorite solution that included the floors, beds, and immediate surrounding of the patients. This was done twice daily. In case of visible dirt, this was first removed with a brush and water, before the application of the sodium hypochlorite solution. The intensive procedure as described in phase 2 (see above) was repeated every two weeks in this phase. The curtains between beds were refreshed every 1-2 months or immediately after visible soiling. In addition, the following IPC measures were taken:

- All patients found positive for one or more carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex, *K. pneumoniae,* or *P. aeruginosa* were cohorted in one dedicated corner of the ICU (see supplemental figure 1). HCWs donned mask, gown, and gloves when approaching and providing care for cohorted patients.
- Finally, in this phase, we introduced for all patients daily total body-washing with cloths soaked in a chlorhexidine gluconate 2% solution. These cloths were prepared and pre-packaged individually in sealed plastic bags by the hospital pharmacy. Oral decontamination with chlorhexidine gluconate 0.5% solution (Minosept®, Minorock) was already a standard

procedure. In the framework of this study a 2% chlorhexidine gluconate solution was introduced in this phase. Bottles containing this solution were also prepared and provided by the hospital pharmacy (see Supplementary Figure 2), and used per patient.

During phase 3, the systematic screening as described for phase 1 was resumed (Figure 1).

Statistical analysis

The description of patients' baseline characteristics and the comparisons of the characteristics between phase 1 and phase 3 were analysed using Chi square or Fisher's Exact and Mann-Whitney tests in SPSS Version 24.0 (SPSS, Chicago, IL, USA). The primary outcome of interest in the study was weekly acquisition of carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex*, K. pneumoniae,* and *P. aeruginosa* per 100 patient-days at risk. We assessed outcomes with a Poisson segmented regression analysis, the parameters of interest were step changes in the acquisition rate per 100 patient-days at risk and changes in trends of acquisition rates per 100 patient-days at risk from phase 1 to phase 3, the ICU number was also included in the model, the final model is shown below:

$$E(y_t) = \mu_t,$$

$$log\mu_t = \beta_0 + \beta_1 \times time_t + \beta_2 \times ICU + \beta_3 \times phase_t + \beta_4 \times timeafterintervention,$$

where y_t represents the weekly acquisition per 100 patient-days at risk at week t, μ_t is the mean of y_t, β_1 is the time trend in phase 1, β_2 the effect of ICU, β_3 is the step change of acquisition form phase 1 to phase 3, β_4 denotes the change in trend of acquisition rate per 100 patient-days at risk from phase1 to phase 3. This statistical analysis was performed using R 3.6.1.(R foundation, R-project.org). The threshold for statistical significance was set at p = 0.01.(11)

Ethics and regulatory considerations

- The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the research on 17th September 2012, No: 561/PT02.FK/ETIK/2012, No: 757/UN2.F1/ETIK/X/2014.
- A Material Transfer Agreement (MTA) was reviewed and approved by the Director of National Institute Research and Development, Ministry of Health (No: LB.02.01/I.9.4/8500/2013).
- Trial registration**:** The study was registered at Netherlands Trial Register http://www.trialregister.nl (No: 5541). Candidate number: 23527, NTR number: NTR5541, NL number: NL5425 (https://www.trialregister.nl/trial/5424), retrospectively registered: NTR: 22 December 2015

RESULTS

A total of 748 patients were enrolled during phase 1(387 patients) and phase 3 (361 patients) of the study. In phase 2, eligible patients were not enrolled in the study except for the first month of the study period when 25 patients were enrolled inadvertently (Figure 1). Since the first intervention, i.e. the hand hygiene promotion program had already started on July 10, 2014, the data of these 25 patients were not included in the analysis of the effect of the bundle of interventions. The patients' characteristics, underlying diseases, length of stay, mortality and other risk factors of patients enrolled in phase 1 and phase 3 are summarized in Table 1.

The comparison of patient characteristics in phase 1 versus phase 3 suggests that the risk of acquisition of carbapenem-non-susceptible strains of nosocomial pathogens, including the three species targeted in this study, may have been higher in phase 3 compared to phase 1. Patients enrolled in phase 3 were more severely ill at the time of their ICU admission, they required more days of mechanical ventilation and, concomitantly, had more central venous catheter and urinary catheter days, and they stayed longer in the ICUs. A higher fraction of the patients in phase 3 were prescribed carbapenem antibiotics as well. Furthermore, shifts in hospital policies favoring short stay (<48 h) of postoperative surgical patients in these ICUs resulted in relatively more medical patients to be enrolled in phase 3 of the study, such patients had another spectrum of underlying diseases (especially more diabetes, less malignancies). In phase 3, the daily oral application and body washes with the 2% chlorhexidine solution were well tolerated, as no lesions of the skin or the oral mucosa were reported by the attending personnel.

We obtained at least two rectal swabs and two throat swabs from each patient.
We analyzed 4,219 screening swabs in total and processed 287 clinical specimens. At admission to ICU, 98 patients were already colonized with carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex, 46 were already colonized with carbapenem-non-susceptible *K. pneumoniae*, and 34 patients were colonized with carbapenem-non-susceptible *P. aeruginosa*. Thus, the large majority of enrolled patients were, at admission, still at risk of acquiring a carbapenem-non-susceptible strain of one or more of the three targeted bacterial species. In phase 1, the acquisition rates were highest for resistant *A. baumannii-calcoaceticus* complex (4.4/100 days at risk), followed by resistant *K. pneumoniae* (2.2/100 days at risk) and resistant *P. aeruginosa* (1.9/100 days at risk) (data not shown). Environmental screening in phase 1 and phase 2 yielded nine isolates of carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex, four carbapenem-non-susceptible *K. pneumoniae*, and 15 carbapenem-non-susceptible *P. aeruginosa* (see supplementary table). We screened 167 healthcare workers once in phase 1 and found only one to carry a carbapenem-non-susceptible *A. baumannii* in the throat.

Table 1. Baseline characteristics of patients admitted to ICUs in phase 1 and phase 3, and enrolled in this study.

Characteristics	Phase 1	Phase 3
Number of patients enrolled	387	361
Age (years); median (IQR)	46 (33-58)	49 (34-69)
Gender		
Male (%)	200 (51.7)	193 (53.5)
Female (%)	187 (48.3)	168 (46.5)
ICU*		
Adult ICU	182 (47.0)	133 (36.8)
ER-ICU	205 (53.0)	228 (63.2)
Underlying diseases		
Cardiovascular (%)	23 (5.9)	30 (8.3)
Cerebrovascular (%)	28 (7.2)	23 (6.4)
Chronic kidney disease (%)	25 (6.5)	10 (2.8)
Diabetes mellitus (%)*	29 (7.5)	52 (14.4)
Malignancy (%)*	111 (28.7)	59 (16.3)
Indication for ICU admission*		
Medical (%)	129 (33.3)	166 (46.0)
Surgical (%)	258 (66.7)	195 (54.0)
Referral from*		
Other ward this hospital (%)	217 (56.1)	138 (38.2)
Other hospital (%)	70 (18.1)	57 (15.8)
Directly from Emergency Unit (%)	100 (25.8)	166 (46.0)
Antibiotic exposure (before admission to ICU)		
Any antibiotic (%)*	300 (77.5)	226 (62.6)
Carbapenem (%)	75 (19.4)	92 (25.5)
SIRS Score (%)*		
Score ≥2	32 (8.3)	102 (28.3)
Score <2	355 (91.7)	259 (71.7)
qSOFA Score (%) *		
Score ≥2	74 (19.1)	197 (54.6)
Score <2	313 (80.9)	164 (45.4)
Procedures (during ICU admission)		
Mechanical ventilation (%)	352 (91.0)	330 (91.4)
Mechanical ventilation duration*		
≥5 days (%)	168 (43.4)	207 (57.3)
<5 days (%)	219 (56.6)	154 (42.7)
Central venous catheter (%)	341 (88.1)	315 (87.3)

Characteristics	Phase 1	Phase 3
Central venous catheter duration*		
≥5 days (%)	205 (53.0)	237 (65.7)
<5 days (%)	182 (47.0)	124 (34.3)
Urinary catheter (%)	387 (100)	361 (100)
Urinary catheter		
≥5 days (%)	232 (59.9)	247 (68.4)
<5 days (%)	155 (40.1)	114 (31.6)
Antibiotic therapy (during ICU admission)		
Any antibiotic (%)	381 (98.4)	348 (96.4)
Carbapenem (%) *	188 (48.6)	224 (62.0)
Outcomes		
Length of stay (days); median (IQR)*	5 (3-9)	7 (4-13)
Death in ICU (%) *	110 (28.4)	137 (38.0)

Abbreviations: ER-ICU. Emergency Room Intensive Care Unit; ICU, Intensive Care Unit; IQR, Interquartile range; qSOFA, quick Sepsis-related Organ Failure Assessment; SIRS, Systemic Inflammatory Response Syndrome.

*p <0.01 when comparing phase 1 versus phase 3.

For all three species taken together there was a significant step change, from phase 1 to phase 3 in the rate of acquisition of carbapenem-non-susceptible strains, the incidence rate ratio (IRR) was 0.374 (CI_{99}: 0.178-0.785) for phase 3 compared to phase 1 (Table 2, Figure 2, panel A). This significant decrease in the overall acquisition rate of carbapenem-non-susceptible strains of the three species was mainly caused by a decrease in the acquisition of carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex (IRR 0.438, CI_{99} 0.181-1.061) and, possibly, some contribution by a lower acquisition rate of carbapenem-non-susceptible *K. pneumoniae* (IRR 0.346, CI_{99} 0.091-1.31) (Table 2, Figure 2). In contrast, within each of the two phases there was no major downward or upward trend observed in the rate of acquisition of resistant strains for any of the three species separately nor for the three species taken together, although the analysis showed that during phase 1 the risk of acquisition was increasing slightly for all three species taken together and for *K. pneumoniae* separately (Table 2).

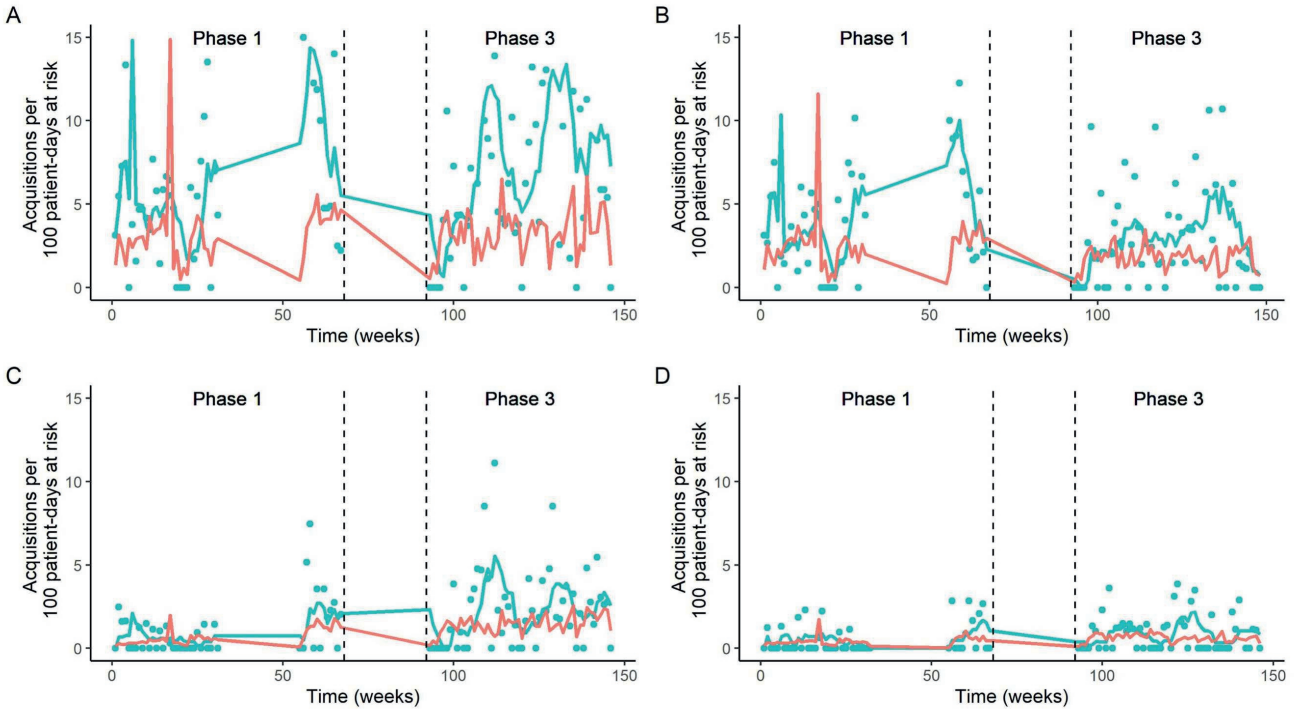

Figure 2. Acquisition of carbapenem-non-susceptible bacteria per 100 patient-days at risk in both ICUs.

Legend: For strains of all three species together (A), for carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex (B), for carbapenem-non-susceptible strains of *K. pneumoniae* (C), and for carbapenem-non-susceptible strains of *P. aeruginosa* (D). The end of phase 1 and the start of phase 3 are marked with vertical dashed lines. Green dots represent weekly acquisition data; green lines are 7-week moving averages. Pink lines are expected values from the Poisson segmented regression model.

Table 2. Trends and step changes in the acquisition rate of carbapenem-non-susceptible strains of *A. baumannii-calcoaceticus* complex, *K. pneumoniae*, and *P. aeruginosa*

	Overall	Carb_NS.*A. baumannii*	Carb_NS.*K. pneumoniae*	Carb_NS.*P. aeruginosa*
Time trend in Phase 1	1.013 (1.003-1.022; p = 0.001)	1.008 (0.997-1.019; p = 0.064)	1.028 (1.007-1.049; p = 0.001)	1.013 (0.987-1.039; p = 0.204)
Step change in Phase 3	0.374 (0.178-0.785; p = 0.001)	0.438 (0.181-1.061; p = 0.016)	0.346 (0.091-1.31; p = 0.04)	0.759 (0.119-4.84; p = 0.701)
Time trend change in Phase 3	0.998 (0.982-1.014; p = 0.702)	0.99 (0.97-1.009; p = 0.168)	0.982 (0.955-1.01; p = 0.1)	0.983 (0.945-1.022; p = 0.25)
Time trend in Phase 3	1.01 (0.997-1.023; 1.02 p = 0.043)	0.997 (0.981-1.014; p = 0.668)	1.009 (0.991-1.029; p = 0.195)	0.995 (0.967-1.025; p = 0.683)

Note: data are incidence rate ratio's with 99% confidence intervals in brackets as assessed by Poisson regression analysis (see Methods)

DISCUSSION

This study showed that introducing a relatively simple and inexpensive bundle of infection prevention and control measures may well reduce the risk of patients admitted to adult ICU in lower-middle income countries to acquire highly resistant strains of common nosocomial pathogens. The bundle consisted of improving hand hygiene practices, reducing contamination of the ICU environment, daily disinfection of patients' skin and oral mucosa, and contact precautions for and cohorting of patients found to carry such strains was particularly effective in reducing the acquisition of carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex, the most prevalent species responsible for such acquisitions in our setting. The bundle was also effective in reducing the acquisition of resistant strains of *K. pneumoniae*, but did not affect *P. aeruginosa* acquisition. The true impact of the bundle may have been even higher, since patients in the third phase of the study were more severely ill and stayed longer in the ICU. In addition, a larger fraction of the patients in phase 3 were prescribed carbapenems. All and all this strongly suggests that patients in phase 3 were more prone to acquire carbapenem-non-susceptible strains than patients in phase 1. However, significantly lower rates of acquisition were observed in phase 3. We did not attempt to perform a risk adjusted analysis of the effect of the intervention since such analysis was not planned à priori, is complicated (due to unknown relationships between parameters and outcome) and may lead to overestimation of the effect of the intervention.

At this time, we cannot offer a solid explanation why especially *A. baumanni-calcoaceticus* complex and not *P. aeruginosa* acquisitions were impacted by our multimodal intervention. Clearly, the rates of acquiring carbapenem-non-susceptible strains were initially much higher in case of *A. baumannii-calcoaceticus* complex than for *P. aeruginosa* (4.4 versus 1.9/100 days, respectively). Thus, there was less opportunity for *P. aeruginosa* strains to become significantly affected by the intervention. The main risk factors for acquisition of a carbapenem-resistant *P. aeruginosa* are, according to a systematic review by Voor in 't holt *et al*., use of carbapenems and medical devices (12). These factors were not targeted in our bundle and both were higher in phase 3. At the genetic level, we, in another publication (13), noted that there was a significant shift in phase 3 in the genotype distribution of the strains circulating in the ICUs, which may have been induced by the intervention, possibly related to the varying susceptibility to sodium chlorite or chlorhexidine among *P. aeruginosa* strains. Alternatively, the sources and transmission routes of *P. aeruginosa* may differ from those of *A. baumannii-calcoaceticus* complex in this setting and, therefore, *P. aeruginosa* may have been little affected by the intervention (14).

Few studies in lower-middle income countries have previously implemented interventions to control the spread of MDR pathogens in hospitals, none from Indonesia, the second most populous LMIC following India. Only one other study, performed in Vietnam and published in 2013, aimed to reduce the rate of acquisition of MDR organisms in adult ICUs in LMIC by a multimodal intervention

– improving hand hygiene, combined with antibiotic stewardship; they were partly successful and reduced the rate of acquisition of methicillin-resistant *Staphylococcus aureus* (MRSA, but not of species of Gram-negative bacilli (15). Likewise, an earlier study from another lower-middle income country, the Philippines, but performed in a neonatal ICU, showed a multimodal intervention – hand hygiene, screening patients, renewed ventilator use protocol, antibiotic stewardship, contact precautions for positive patients, daily and monthly infection control checklists - to reduce MRSA colonization only but not to affect the colonization of neonates by resistant Gram-negative bacilli (16) In contrast, Apisarnthanarak *et al*. in Thailand, a upper-middle income country in the same region of the world as Indonesia, applied in a quasi-experimental study a multimodal intervention – hand hygiene, screening and cohorting positive patients, and environmental disinfection with sodium hypochlorite – in 3 adult ICUs and found it to be effective in reducing the colonization and/or infection with drug-resistant strains of *A. baumannii* (17); other species of drug resistant pathogens were not targeted in that study.

In our bundle, much emphasis was placed on reducing contamination of the ICU environment, since the ICU environment has been shown to provide niches for the three species of nosocomial pathogens targeted (12, 14) We now feel that assuring a clean ICU environment, including access to safe and clean water and water systems (sinks), is important and should be included in intervention bundles aimed to reduce the acquisition of MDR strains in ICUs. The bundle we designed was low cost and simple to apply. Hand hygiene compliance can be improved significantly in ICUs in other lower-middle income countries.(18-21) As with any measure, hand hygiene and environmental stewardship require a systematic approach and should be built into ICU protocols, effectively reinstating environmental cleanliness as a prime infection control issue.(22) Although introducing multifaceted hand hygiene programs have been shown to be efficacious in studies in lower-middle income countries, including Indonesia (10, 23), observation of clinical practice has shown compliance to hand hygiene to fall back easily after hand hygiene improvement program has stopped.(10, 18)Thus, a systematic permanent program to maintain hand hygiene compliance is needed. Similarly, the crucial role of the environment of the ICU should be translated into and become part of the infection control efforts of the ICU, effectively regarding cleaning service personnel as important partners of the infection control team.

Originally, we planned to also restrict the use of antibiotics, especially carbapenems for empirical treatment in the ICU, but this proved not to be feasible because alternative regimens were not reimbursed under the national insurance programme or were not available in Indonesia.

We are aware about the potential side effects of chlorhexidine gluconate 2% as mentioned in a recent report by Plantinga et al.(24), but we did not observed and record such adverse events in our patient cohorts, possibly due the fact that such side effects are observed only after prolonged use of chlorhexidine (> 21 days), which is longer than we applied chlorhexidine in our patients.(24)

The study has several limitations. First, it was a single centre study precluding extrapolation of our findings to other ICUs in Indonesia and other lower-middle income countries. However, much may be relevant and learned from our experience. Second, the quasi-experimental design has its limitations. In our case, potential negative confounding was introduced by significant differences in patient profiles between the two study phases, mostly due to uncontrollable managerial policy changes. Multicentre, cluster randomized studies may provide a more robust design, and produce results that can be extrapolated (25), although these would be much more expensive and more difficult to manage in lower-middle income countries. Finally, not all independent parts of the bundle were systematically checked for compliance. For the hand hygiene improvement strategy, this was done as described previously.(10) For the chlorhexidine bathing and mouth wash and cohorting, this was executed well for each patient, but not recorded. The contact precautions were more difficult to implement, as the personal protective equipment had to be paid by the patient, which was sometimes not possible. The added value of contact precautions in our bundle may therefore be questioned. The environmental program was implemented well, and this was regularly checked by the authors and the ICU nurses, but not recorded. In a follow-up study, an auditing system should be used to monitor this.

CONCLUSION

We conclude that a multimodal intervention aiming to prevent acquisition of resistant strains of important ICU pathogens is feasible and may be quite effective in ICUs in lower-middle income countries. Environmental cleaning should be an important part of the intervention. To control MDR *P. aeruginosa*, however, additional strategies should be explored.

DECLARATION

Ethics and regulatory considerations

- The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the research on 17th September 2012, No: 561/PT02.FK/ETIK/2012, No: 757/UN2.F1/ETIK/X/2014.
- A Material Transfer Agreement (MTA) was reviewed and approved by the Director of National Institute Research and Development, Ministry of Health (No: LB.02.01/I.9.4/8500/2013).
- Trial registration**:** The study was registered at Netherlands Trial Register http://www.trialregister.nl (No: 5541). Candidate number: 23527, NTR number: NTR5541, NL number: NL5425 (https://www.trialregister.nl/trial/5424), retrospectively registered: NTR: 22 December 2015

Consent for publication

Informed consent was documented by the use of a written consent form approved by the Ethics Committee Faculty of Medicine Universitas Indonesia / Dr. Cipto Mangunkusumo General Hospital and signed and dated by the subjects/guardians and by the person who conducted the informed consent discussion and two witnesses. The signature confirmed the consent was based on information that had been understood.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

YRS is an awardee of the DIKTI-NESO Scholarship by The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, and Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands.

All authors report no conflict of interest relevant to this article.

Funding*:*

This work was supported by 'The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia' and 'Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands'.

Authors' contributions

YRS, AK, HAV, and JAS conceived the study and participated in design of the study.

YRS, RS, and DA participated in acquisition of data.

YRS, HQ, HAV, and JAS performed data analysis and interpreted the data.

YRS, HQ, HAV, and JAS drafted the article.

All authors participated in critically revising the draft.

All authors read and approved the final manuscript.

Acknowledgements

We are thankful to The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, Dean of Faculty of Medicine Universitas Indonesia, Board of Directors of Dr. Cipto Mangunkusumo National General Hospital Jakarta Indonesia, Department of Medical Microbiology and Infectious Diseases, Erasmus

MC in Rotterdam, The Netherlands, and Critical Care Division, Department of Anesthesia and Intensive Care, Faculty of Medicine Universitas Indonesia / Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia, for facilitating this research. We also thank all nurses, physicians and cleaning personnel of the adult ICU and ER-ICU of Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia, for their contribution. Ns Sondang Sihite (Infection Prevention Control Nurse adult ICU), Ns Eliyani (Infection Prevention Control Nurse ER-ICU) are gratefully acknowledged for their involvement. Pharmacist Unit of Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia, for their contribution to in house made of chlorhexidine gluconate 2 % solution and body wipes. We thank Dr. Joost van Rosmalen from Department of Biostatistics, Erasmus MC University Medical Center, Rotterdam, The Netherlands, for his technical advice in the early stages of this work.

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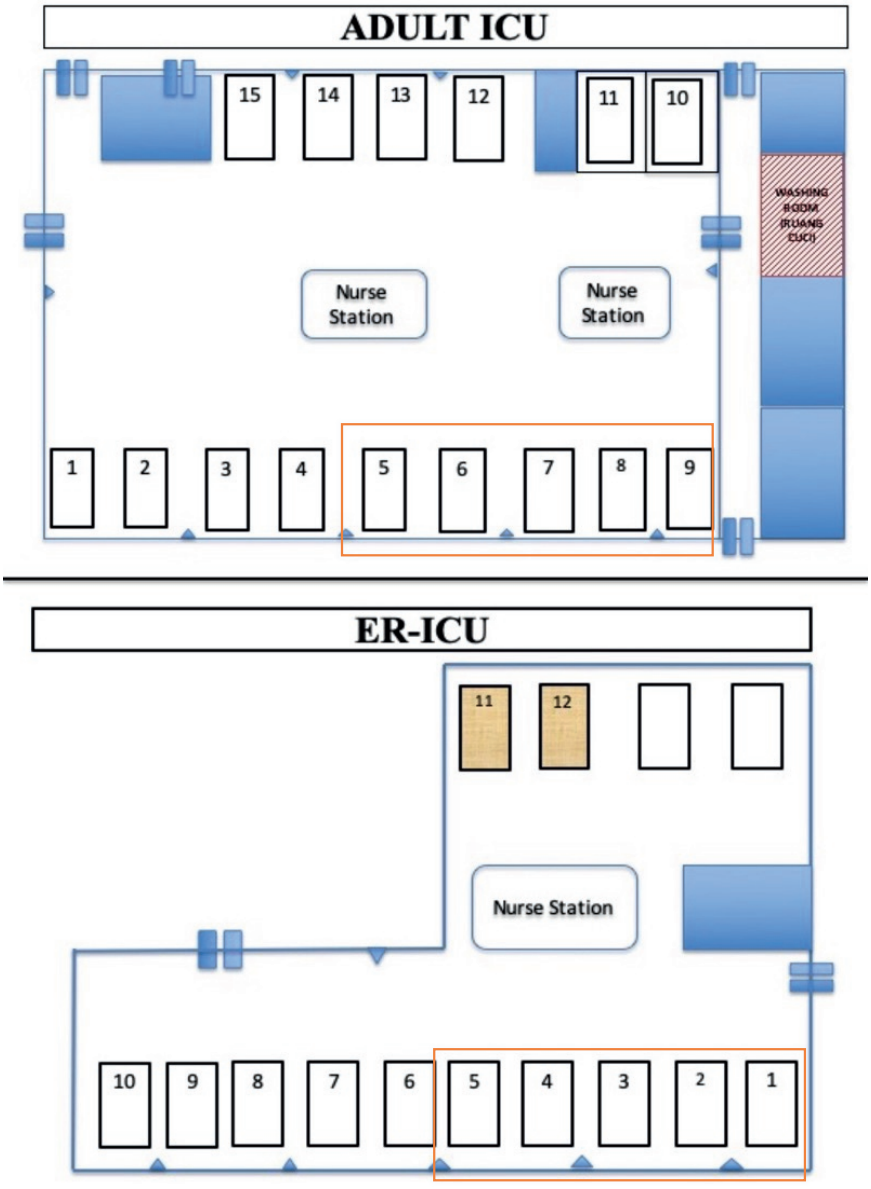
SUPPLEMENTAL MATERIALS

Supplementary Table 1. List of environmental samples

Sample site	Number of samples	
	Adult ICU	ER-ICU
Wash basin on ICUs ward	10	5
Monitor	14	10
Ventilator	15	5
Ambu bag	8	
Stethoscope	10	9
Drawer handle bedside cabinet	21	
Plastic multi-purpose container next to each bed	18	8
Stainless steel container	8	15
Flowmeter	15	
Infusion stand	11	8
Infusion pump	9	6
Bed rails	20	14
Tap water (wash basin on ICU ward)	10	8
Chart paper on bedside cabinet	11	6
Bedside cabinet table	15	11
Cleaning room: wash basin	9	
Cleaning room: sink countertop	3	
Cleaning room: mug	2	
Cleaning room: dish rack	3	
Mattress	5	4
Comb	3	
Water from siphon of wash basin	10	5
Water from mug next to each bed	10	6
Massage oil	5	
Chlorine solution after use	3	
Cleaning wipes	3	4
Wall	3	2
Drawer of bedside cabinet	3	
Water from suction	7	
Suction connector/container	3	
Water from humidifier	1	
Water after cleaning a floor	2	
Floor	2	
Nurse station	1	1

Abbreviations: ER-ICU. Emergency room Intensive Care Unit; ICU, Intensive Care Unit



Supplementary Figure 1. ICUs layout.

Legend: ICUs layout, the black square indicated as a bed, the red square line indicated as a cohorting place. In ER-ICU, there were 2 yellow bed, that use only in phase 3

Supplementary Figure 2. Chlorhexidine gluconate 2% solution and Body wipes

CHAPTER 9

9

High-risk international clones of carbapenem non-susceptible *Pseudomonas aeruginosa* endemic in Indonesian intensive care: impact of a multifaceted infection control intervention analyzed at the genomic level.

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ABSTRACT

Infection control effectiveness evaluations requires detailed epidemiological and microbiological data. We analyzed the genomic profiles of Carbapenem Non-susceptible *Pseudomonas aeruginosa* (CNPA) strains collected from two intensive care units (ICUs) in the national referral hospital in Jakarta, Indonesia, where a multifaceted infection control intervention was applied. We used clinical data combined with whole-genome sequencing (WGS) of systematically collected CNPA to infer CNPA strain's transmission dynamics and characterize their resistome. We found that the number of CNPA transmissions and acquisitions by patients was highly variable over time but that overall the rates were not significantly reduced by the intervention. Environmental sources were involved in these transmissions and acquisitions. Four high-risk international CNPA clones (ST235, ST823, ST357 and ST446) dominated, but the distribution of these clones changed significantly after the intervention was implemented. Carbapenem resistance was explained from resistome analysis by the presence of various carbapenemase-encoding genes ($bla_{\text{GES-5}}$, $bla_{\text{VIM-2-8}}$, $bla_{\text{IMP-1-7-43}}$) and by mutations within the porin OprD. Our results describe for the first time the dynamics of *P. aeruginosa* AMR profiles in Indonesia, and additionally shows the utility of WGS in combination with clinical data to evaluate the impact of an infection control intervention.

IMPORTANCE:

In low-to-middle income countries such as Indonesia, the work in intensive care units (ICUs) can be hampered by lack of resources. Conducting large epidemiological studies in such settings using genomic tools is rather challenging. Still, we were able to systematically study the within and between ICU transmissions of carbapenem non-susceptible strains of *P. aeruginosa* (CNPA), before and after an infection control intervention. Our data show the importance of the broad dissemination of the internationally recognized CNPA clones, the relevance of environmental reservoirs, and mixed effects of the implemented intervention: it led to a profound change in the clonal make-up of CNPA, but it did not reduce the patients' risk of CNPA acquisitions. Thus, CNPA epidemiology in Indonesian ICUs is part of a global expansion of multiple CNPA clones that remains difficult to control by infection prevention measures.

KEYWORDS:

Microbial drug resistance, *Pseudomonas aeruginosa*, Intensive Care Units, infection control, Single Nucleotide Polymorphism, Indonesia.

INTRODUCTION

Pseudomonas aeruginosa is especially dreaded as one of the leading species to cause healthcare-associated infections [1,2]. *P. aeruginosa* is an opportunistic human pathogen with a remarkably versatile genome, which allows it to adapt to a wide range of environments and conditions and, consequently, survive in a variety of niches. This is mainly due to traits encoded in its accessory genome, which includes genes coding for antimicrobial resistance (AMR), a great diversity of metabolic pathways, and virulence factors [3]. AMR is a major concern in clinical *P. aeruginosa* isolates, as almost 31% of all invasive isolates are resistant to at least one of the main antimicrobial groups tested, according to the most recent AMR surveillance report by the European Centre for Disease Prevention and Control (ECDC) [4]. Additionally, a limited number of *P. aeruginosa* clones with multi-drug resistance (MDR) profiles are particularly worrisome since they have been shown to have achieved nearly global expansion [5,6]). Especially in low- to middle-income countries, MDR *P. aeruginosa* contributes to in-hospital mortality [7,8]. Gathering as much clinical and microbiological information as possible with respect to these isolates is essential to inform nosocomial infection control and surveillance procedures.

During recent years, whole-genome sequencing (WGS) has developed rapidly into a reference tool for outbreak management [9–12]. However, there is not a common standardized and accepted methodology to infer bacterial transmissions during outbreak investigations from WGS data. This is troublesome, especially when the WGS approach is implemented in regions of the world where MDR and XDR microorganisms are already endemic.

The aim of this study was to assess nosocomial transmission of carbapenem non-susceptible *P. aeruginosa* (CNPA) using WGS in combination with detailed clinical data, from ICU patients of the national referral hospital of Indonesia. CNPA were systematically collected before and after an infection control intervention, such that we could study its effect on the dynamics of transmission of CNPA in this setting in detail. Additionally, we highlight the main *P. aeruginosa* clones found as well as their resistomes. Risk factors for the carriage and acquisition of CNPA and its effect on patients' outcomes have been analyzed and published separately [13].

Study design – sample collection

We performed a prospective, quasi-experimental before-and-after study in two ICUs of the national referral hospital of Indonesia. Dr. Cipto Mangunkusumo Hospital is a 1200-bed university hospital located in Jakarta. We conducted this study in two ICUs for adult patients: the 12-bedded adult ICU and the 8-bedded Emergency Room (ER)-ICU with an average of 1,010 and 415 admissions per year, respectively. Both ICUs have an open ward design. The populations served by these two ICUs were very similar, and there was also no difference in the care provided [8]. The study consisted of three study phases, pre-intervention phase (April-October 2013 and

April-August 2014), intervention phase (December 2014- January 2015) and post-intervention phase (February-December 2015) [8,14].

CNPA strains were collected from clinical cultures and by targeted screening in the pre- and post-intervention phases. Healthcare personnel and the ICU environment were screened for CNPA as well, once in both pre- and post-intervention phases of the study. A list of the isolates together with clinical data, can be found in **Table S5**. All isolates were stored in 10% glycerol containing media and frozen at -80°C until further use. The study was registered at http://www.trialregister.nl (Trial NL5424 (NTR5541)). Further details on the wards for the period 2013-2014, the sampling process and microbiological methods such as the CNPA selection criteria, have been previously described in detail [8]. Replicate collections of all these isolates were archived in Jakarta (Indonesia), Rotterdam (The Netherlands) and La Balme Les Grottes (France).

Intervention

Between the two collection periods mentioned above, an infection control bundle aimed at reducing transmission of carbapenem-non-susceptible *P. aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii-calcoaceticus* complex was implemented in both ICUs. The measures adopted with this intervention included enhanced environmental cleaning, enforced antibiotic stewardship which included a daily revision of all antibiotics on weekdays, and a targeted hand hygiene education for healthcare workers of the ICUs [14]. Once-daily bathing with chlorhexidine 2% was introduced and for intubated patients, oral hygiene was performed four times per day by rinsing with 2% chlorhexidine solutions. Patients colonized or infected with carbapenem non-susceptible Gram-negative bacteria were grouped together in a dedicated area of the ward, with contact isolation precautions as recommended by the CDC.
(https://www.cdc.gov/infectioncontrol/basics/transmission-based-precautions.html).

Bacterial identification, antibiotic susceptibility testing and DNA extraction

Stored strains were regrown from the -80°C stocks using Columbia agar + 5% sheep blood (COS plates; bioMérieux, Marcy-l'Étoile, France) and colonies confirmed to be *Pseudomonas aeruginosa* using Vitek® MS with standard acquisition parameters according to the manufacturers' instructions (bioMérieux). Antibiotic susceptibility testing (AST) was performed with Vitek® 2 (bioMérieux) using EUCAST 2019 breakpoints [15]. The antibiotics tested were ticarcillin, piperacillin, ticarcillin/clavulanic acid, piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, aztreonam, ciprofloxacin, levofloxacin, amikacin, gentamicin and tobramycin. Susceptibility to colistin was not reported, since a validated automated test to do so was lacking.

Whole Genome Sequencing and Bioinformatics

DNA was extracted from pure cultures using the UltraClean® microbial DNA isolation kit (Qiagen N.V., Venlo, Netherlands) and quantity and quality were assessed using the Qubit dsDNA BR assay kit (ThermoFisher Scientific, Waltham, MA USA).

(i) Whole-genome sequencing methods and quality control.

Samples from the pre-intervention phase were sequenced using either a HiSeq 2500 instrument (Illumina Inc., Cambridge, United Kingdom) with 150-bp paired-end reads or the MiSeq instrument (Illumina Inc.) with 200-bp paired-end reads. Samples from the post-intervention phase were sequenced using a NextSeq 500 (Illumina Inc), with 150-bp paired-end reads. Nextera XT DNA library prep kit (Illumina Inc.) was used in all cases. Paired-ended reads were assembled into contigs and scaffolds using the A5-MiSeq pipeline (v20160825) [16]. Correct identity of assemblies was confirmed by an Average Nucleotide Identity (ANI) analysis using FastANI (v1.2) [17], using *P. aeruginosa* PAO1 as reference (NC_002516.2). QUAST (v5.0.2) was run to assess the assemblies quality, using standard parameters and including the "–scaffold" parameter when scaffolds were obtained from the assembly [18]. Reads and assemblies from all sequenced samples are available at the European Nucleotide Archive website under the project name PRJEB30625 and PRJEB32907, for the clinical and environmental samples, respectively.

(ii) Antimicrobial resistance and MLST typing.

Antimicrobial resistance determinants were identified from assemblies using the Resistance Gene Identifier (RGI) command line tool associated with the Comprehensive Antimicrobial Resistance Database [19–21] (updated in 2018, analysis date: December 2018), using the "Strict algorithm", which allows the detection of previously unknown AMR genes. To display the results of the RGI tool, we used a cluster map created with a custom Python 3 script. Additionally, we screened the literature for genes belonging to the mutational resistome of *P. aeruginosa*, and analyzed them using Snippy (v1.4.1) (Seemann T [2015] snippy: fast bacterial variant calling from NGS reads. https://github.com/tseemann/snippy) by aligning the contigs to the *P. aeruginosa* PAO1 reference genome (NC_002516.2), and selecting missense variants with a minimum coverage of X50. BioNumerics® 7.6 (Applied Maths, St-Martens-Latem, Belgium) was used for *in silico* Multi Locus Sequence Typing (MLST) using the pubMLST database site (hosted at https://pubmlst.org by the University of Oxford). goeBURST was used to infer the relatedness of the MLST profiles [22].

(iii) Genomic epidemiology of the bacterial strains.

We used the assemblies to perform a *k*-mer based SNP analysis using kSNP3 (v3.01) [23]. kSNP3 was executed with the parameters "–k 21 –core", setting the *k*-mer nucleotide length to 21-

bp and allowing the calculation of the "core SNPs". The "core SNPs" are those identified from *k*-mers present in all input samples. The goal was to use SNPs data and the available clinical information to infer transmission patterns of individual CNPA strains between patients during the whole study period. To do so, the first step was to set up a similarity SNP cut-off, below which all isolates were considered genetically identical, i.e. isogenic. From all kSNP3 output files, we used the file containing the *k*-mer core SNPs detected ("core_SNP_matrix.fasta"), and used it as input file for snp-dists (v0.6; https://github.com/tseemann/snp-dists). snp-dists was executed with the "–a –b" parameters. We used the pairwise SNP matrix to evaluate different SNP thresholds. To calculate the optimal SNP threshold value, we assumed that truly identical strains could only be cultured from the same patient, and that different patients never shared the same strain. Thus, we considered a true positive (TP) match when two isolates from the same patient had a number of SNP differences below or equal to the tested threshold, a true negative (TN) when two isolates from different patients had a number of SNP differences above the tested threshold, a false positive (FP) when two isolates from different patients had a number of SNP differences below or equal to the tested threshold, and a false negative (FN) when two isolates from the same patient had a number of SNP differences above the tested threshold. Using a custom Python script we counted the number of true positives (TP), true negatives (TN), false positives (FP) and, false negatives (FN), and then calculated sensitivity and specificity values for a large range of thresholds (0 - 20000). Sensitivity and Specificity values for these different SNP thresholds were used to calculate and plot a cumulative distribution analysis figure and Receiver Operating Characteristic (ROC) curves to finally select the optimal similarity SNP cut-off using Youden's Index. Isolates that had SNP profile differences below this cut-off were considered to be isogenic, i.e. represent the same strain circulating in this ICU setting at the time of the study. We also evaluated the effect of varying the genotype distribution of the CNPA collection on the optimal SNP cut-off value by similarly calculating optimal SNP cut-off values for different sub-collections of our initial panel of *P. aeruginosa* isolates.

(iv) Possible transmission events vs acquisition events.

In order to highlight the importance of clinical metadata in the outcome and interpretation of the genomic epidemiology analysis, we differentiated between two approaches to account for transmissions of CNPA strains in the ICU setting: the first one takes into account only the genetic concordance between isolates based on SNP differences generated by WGS, yielding what we called Possible Transmission Events (PTEs). We defined a PTE as two isolates cultured from two different patients - or a patient and an environmental sample - who were genetically considered to be the same (isogenic) because the SNP profiles of their core genomes differed less than the SNP cut-off value (see above). In the second approach, we incorporated both the genetic

concordance and the clinical and other laboratory information, such as patient ID, date of the culture and patients admission and discharge dates, to generate what we called Acquisition Events (AEVs). A patient was considered to have acquired a CNPA in the ICU only when screening at admission was negative and when the first CNPA was isolated from a sample taken at least 48 hours after admission to the ICU. A patient could have an AEV from either a known or an unknown source. An AEV from a known source was defined when a patient acquired a CNPA strain that was genetically the same (as defined above) as an isolate cultured earlier from another patient or from an environmental site, *and* the clinical and microbiological data (e.g. sampling date) could not exclude that a transmission had occurred between them. If the origin of a CNPA strain cultured from a given patient could not be traced back to a previously identified source, we labelled this AEV as "from an unknown source". To calculate either PTE or AEV, we considered only the first/earliest CNPA strain isolated, subsequent isogenic isolates from the same patient were ignored in enumerating the number of PTEs and AEVs. To compare transmissions before and after the intervention, the occurrence of PTEs and AEVs was expressed as an attack rate, using the total number of patients at risk of CNPA transmission for each period as denominator. Chi-square test was used to report significance.

Finally, as measures of diversity we also calculated the first three Hill Numbers (^0D, ^1D, ^2D) [24] for the CNPA collections cultured in each of the pre- and post-intervention phases. These Hill numbers are mathematically converted classic diversity indexes ^0D = richness, ^1D (exponential of Shannon-Wiener's diversity index), and ^2D (the reciprocal of Gini-Simpson's index).

RESULTS

A total of 412 patients were included in the study during the pre-intervention phase (188 were admitted to the adult ICU and 224 to the Emergency Room-ICU) and from 51 (12.4%) patients at least one CNPA was isolated. During the post-intervention phase 363 patients were included (Adult ICU:133 and ER-ICU: 230), and from 52 (14.3%) patients at least one CNPA was isolated [8]. Risk factors, including antibiotic usage, and patient outcomes of CNPA carriage and acquisition during ICU stay are reported elsewhere [13]. A total of 119 CNPA strains were isolated during the pre-intervention phase, hereby defined as the "pre-intervention phase" set, which included 12 environmental isolates. 118 CNPA strains were isolated during the post-intervention phase, hereby named the "post-intervention phase" set, including 3 environmental isolates. Note that the two strain collections contained multiple CNPA isolates per patient. This was the case for 51 patients. In order to avoid overrepresentation of specific genotypes, in indicated calculations we used only the first isolate per unique genotype per patient (see below).

Phenotypic identification and antibiotic susceptibility testing of the P. aeruginosa strains

Vitek® MS confirmed the correct identification of all *P. aeruginosa* isolates, (data not shown). A total of 130/237 (54.9%) isolates were resistant to all antibiotics tested. Detailed susceptibility results at the isolate level are presented in **Table S1**.

Sequencing statistics and assembly quality

The median N50 value was 225,489 bp (IQR 195,941 – 269,325 bp), and the median contigs number was 114 (IQR 72 – 148) . Genomes sizes ranged from 6,3Mb to 7,3Mb. FastANI identity values for all assemblies were over 98.5%, confirming the correct species nature of all isolates. Detailed sequencing statistics and QUAST [18] results are summarized in **Table S2**. The quality criteria were met for all sequences.

***in silico* MLST**

We identified four major CNPA sequence types (ST235, ST357, ST823, ST446), and three new sequence types (ST3275, ST3277, ST3278), along with 12 minor STs (**Figure 1**). A goeBURST analysis showed that 17/19 of the sequence types found belong to an already existing *P. aeruginosa* clonal complex, while ST1189 and ST3277 were singletons (**Figure 1S**). Between the two phases of the study we observed a clear shift in the sequence type distribution: while in the pre-intervention phase ST235 was the dominant sequence type, in the post-intervention phase ST357 had emerged as the dominant ST. Only the main sequence types and ST244 were present in both phases of the study, the remainder were detected only in the pre-intervention phase (ST2951, ST620, ST274, ST1189, ST253, ST3277 and ST1182) or only in the post-intervention phase (ST3278, ST455, ST1076, ST555, ST312, ST260 and ST3275). Detailed results regarding the MLST profiles of all isolates are filed in **Table S3**.

Analysis of AMR determinants

We found 102 different AMR-related genes, from which at least 32 were acquired resistance genes according to literature. Additionally, 70 genes were analyzed with snippy, which brought to light a considerable amount of mutations directly related to antibiotic resistance (such as the ampC cephalosporinase or penicillin-binding proteins), and mutations in intrinsic genes such as RND efflux pumps and regulators. The mutational resistome obtained with snippy can be found in **Table S4**. **Figure 2A** presents a heat map of the AMR determinants found by RGI-CARD in each genotypically unique strain in the CNPA collection. Eighteen beta-lactam resistance genes were detected, among them genes encoding the carbapenem degrading enzymes *bla*$_{\text{GES-5}}$ (16/130, 12.3%), *bla*$_{\text{IMP-1}}$ (1/130, 0.8%), *bla*$_{\text{IMP-7}}$ (42/130, 32.3%), *bla*$_{\text{IMP-43}}$ (1/130, 0.8%), *bla*$_{\text{VIM-2}}$ (26/130, 20.0%) and, *bla*$_{\text{VIM-8}}$ (1/130, 0.8%). Interestingly, some of these genes were restricted to certain

CNPA clones, including *bla*$_{GES-5}$, *bla*$_{IMP-1}$, *bla*$_{IMP-43}$, *bla*$_{OXA-10}$ and *bla*$_{VIM-8}$ genes that were found only in ST235, while ST823 was the only sequence type harbouring *bla*$_{VIM-2}$. As expected, strains carrying these carbapenemase genes had high imipenem and meropenem MIC values (**Figure 2B**, subplots 1 and 2).

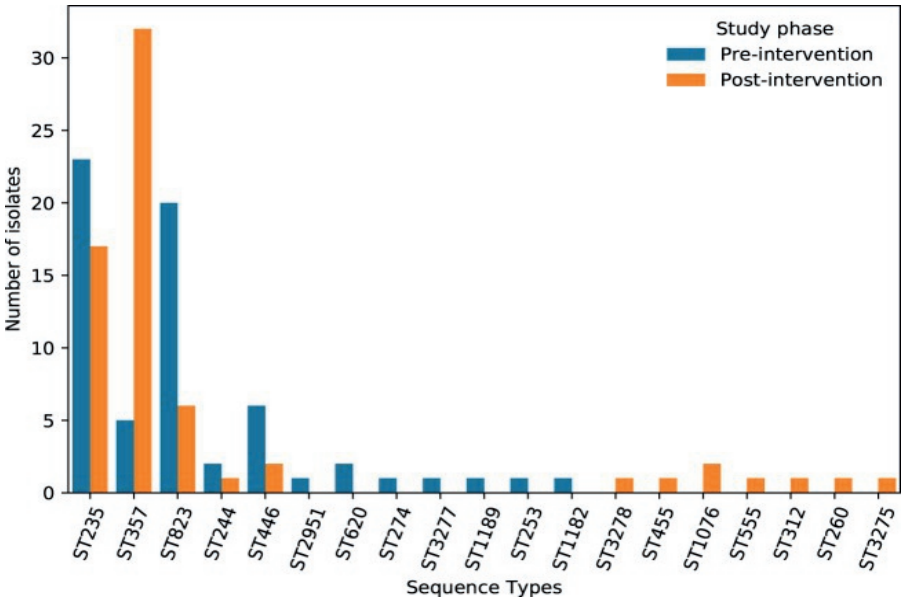

Figure 1. Multilocus sequence type of genotype-corrected CNPA. Sequence types are displayed in the abscissa axis. The ordinate axis indicates the number of genotype-corrected isolates.

The mutational analysis of the oprD revealed 32 different missense mutations, including insertions leading to frameshifts. Only one isolate had an OprD sequence identical to that of the type strain PAO1, the rest accumulated a pattern of point mutations that led to amino acid changes in the primary protein structure of the OprD porin. Eight out of the 32 amino acid substitutions were present in more than 50% of the analyzed isolates: $T_{103}S$ (83/130, 63.9%), $K_{115}T$ (83/130, 63.9%), $F_{170}L$ (82/130, 63.0%), $E_{185}Q$ (118/130, 90.8%), $P_{186}G$ (116/130, 89.2%), $V_{189}T$ (118/130, 90.8%), $R_{310}E$ (92/130, 70.8%) and, $A_{315}G$ (88/130, 67.7%). Detailed results of the amino acid substitutions patterns can be found in **Table S4**. We observed that in carbapenemase non-producing strains, certain OprD types were prone to have higher imipenem and meropenem MIC values (**Figure 2B**), but we could not establish a conclusive correlation between these porin gene mutations and the phenotypic susceptibility patterns of the CNPA strains.

Four different AMR determinants known to confer reduced susceptibility to quinolones were found. The *gyr*A modification that confers resistance to quinolones in *P. aeruginosa* (through the T83I amino acid substitution) was present in 105/130 (80.8%) strains, all of them belonged

to the prevalent clones ST235, ST357, ST823 and to minor clone ST244. The T83I amino acid substitution in *gyrA* was present in all strains that had MICs >= 4 mg/L for ciprofloxacin (**Figure 2B**, subplot 3). Two additional amino acid substitutions were found in this gene (H148N and D682E). Additional mutations were found in the genes *gyrB*, *parC* and *parE* (see **Table S4**). The aminoglycoside resistome of the collection constituted 21 different aminoglycoside-modifying enzymes and their variants, but the most prevalent aminoglycoside resistant determinant was the chromosomally encoded APH(3')-IIb which was present in all strains. Additionally, we also detected mutations in *mexZ* and *fusA1*, both genes previously linked to aminoglycoside resistance [25] (see **Table S4**). We observed that all these genes were associated to variable levels of susceptibility to amikacin (**Figure 2B**, subplot 4). Regarding polymyxin resistance, we did not find any of the plasmid-mediated *mcr* genes. With RGI-CARD we only found three AMR determinants (*arnA*, *basR* and *basS*). *arnA* is a component of the *arnT* operon, but was the only gene of the operon present. The response regulator gene (*basR*) of the two-component regulatory system BasRS was absent in all strains belonging to the prevalent clones ST357 and ST823, and the minor clones ST1076, ST312 and ST3277. We expanded this analysis with the mutational resistome, including other genes related to polymyxin resistance. Finally, genes related to bycyclomycin, fosfomycin and chloramphenicol resistance (bcr-1, fosA, and catB, respectively) were present in all isolates of the collection.

Genomic epidemiology

The optimal threshold for distinguishing isogenic CNPA strains from other strains circulating in this clinical setting was found to be ≤ 5 SNPs difference. Thus, isolates that had core genome SNP profile differences below this threshold were considered to belong to the same, i.e. isogenic, strain present in this clinical setting. This cut-off value had an à priori sensitivity and specificity of 0.76 and 0.95, respectively (**Figure 3**). This 5 SNPs cut-off was compatible with the median SNPs differences found between isolates belonging to the dominant multi-locus sequence types, these median [range] SNP differences were 59 [0-82] for strains belonging to ST235, 17 [0-32] for ST357 strains, 5 [0-54] for ST446 strains, and 5 [0-54] for ST823 strains. We observed several cases where multiple CNPA isolates, cultured from the same patient, had the same multi locus sequence type, but differed by more than the 5 SNPs threshold, indicating that those were different strains circulating independently from each other in this clinical setting at the time of the study. Such finding included cases with isolates that were acquired and those that were imported into the ICU. Also, we observed that excluding prevalent sequence types from the calculations had some impact on the optimal cut-off (**Table S6**), indicating that the genotypic composition of a collection of CNPA isolates may generate (slightly) different optimal cut-off values.

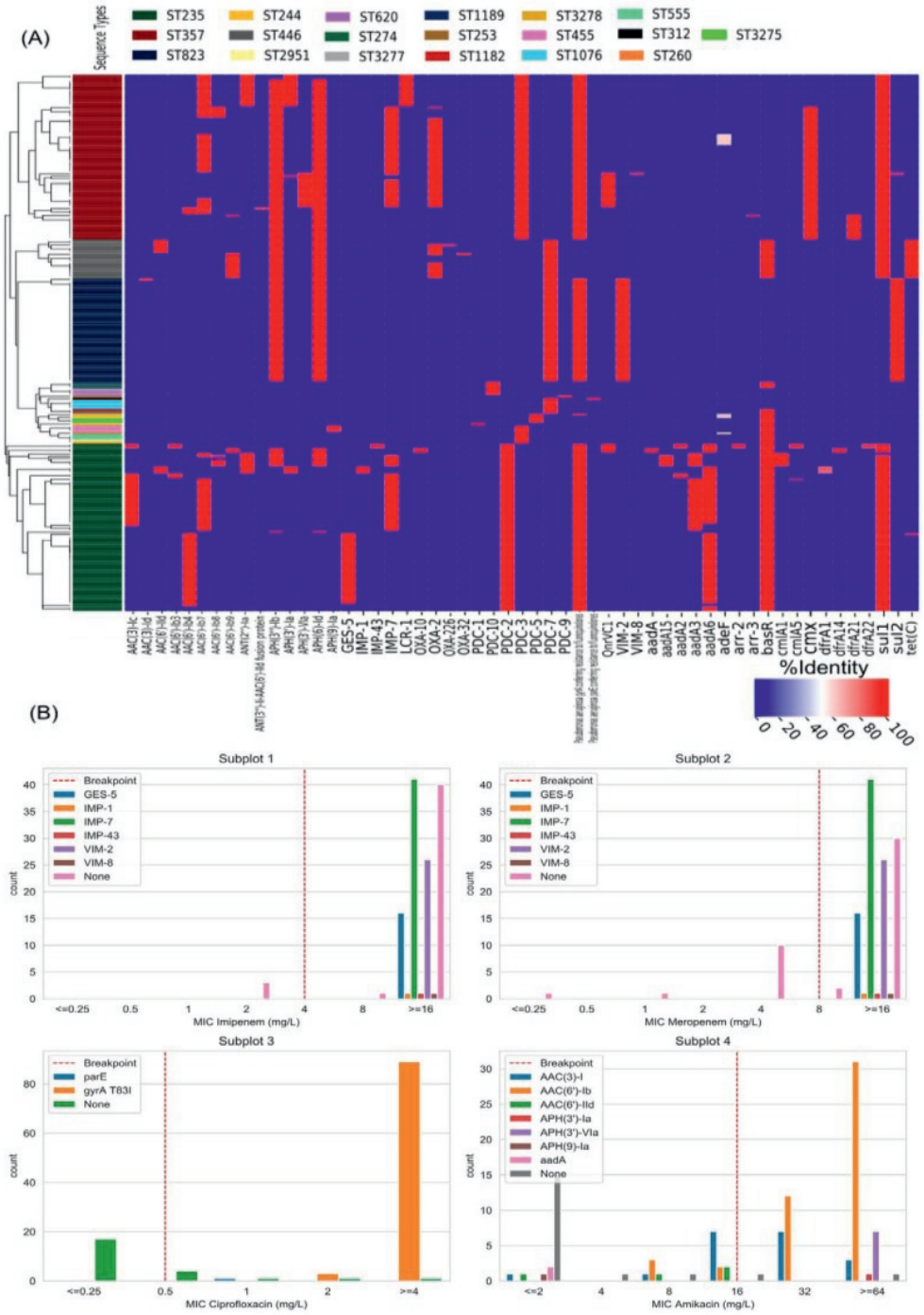
(A)
Sequence Types
ST235
ST357
ST823
ST244
ST446
ST2951
ST620
ST274
ST3277
ST1189
ST253
ST1182
ST3278
ST455
ST1076
ST555
ST312
ST260
ST3275
%Identity
0
20
40
60
80
100
(B)
Subplot 1
Breakpoint
GES-5
IMP-1
IMP-7
IMP-43
VIM-2
VIM-8
None
count
MIC Imipenem (mg/L)
Subplot 2
MIC Meropenem (mg/L)
Subplot 3
parE
gyrA T83I
MIC Ciprofloxacin (mg/L)
Subplot 4
AAC(3)-I
AAC(6')-Ib
AAC(6')-IId
APH(3')-Ia
APH(3')-VIa
APH(9)-Ia
aadA
MIC Amikacin (mg/L)

Figure 2. (A) Antimicrobial resistance heat map of 130 isolates of carbapenem-nonsusceptible *P. aeruginosa* (CNPA). The x axis contains only AMR determinants that were variably present in the CNPA collection. The following AMR determinants are not displayed because they were present in all isolates: APH(3′)-IIb (aminoglycoside resistance); blaOXA-50 (β-lactam resistance); fosA (fosfomycin resistance); bcr-1 (bicyclomycin resistance); arnA and basS (polymyxin resistance); catB (chloramphenicol resistance); pmpM (multidrug and toxic compound extrusion [MATE] transporter); emrE (small multidrug resistance efflux pump); crpP (quinolone resistance); mexA-mexB-oprM plus mexR, nalC, and nalD plus cpxR plus ArmR (resistance-nodulation-cell division [RND] efflux pump plus mexAB repressors plus mexAB activator plus mexR inhibitor); mexC-mexD-oprJ plus NfxB (RND efflux pump plus mexCD-oprJ repressor); mexE-mexF-oprN plus mexT plus mexS (RND efflux pump plus mexEF activator plus mexT suppressor); mexG-mexH-mexI-opmD plus soxR (RND efflux pump plus transcriptional activator); mexJ-mexK-opmH plus mexL (RND efflux pump plus mexJK repressor); mexM-mexN-oprM (RND efflux pump); mexP-mexQ-opmE (RND efflux pump); mexV-mexW-oprM (RND efflux pump); muxA-muxB-muxC-opmB (RND efflux pump); triA-triB-triC-opmH (RND efflux pump); mexY plus mexZ (RND efflux pump component plus mexXY transcriptional regulator). (B) Bar plots showing the relation between the MIC of imipenem, meropenem, ciprofloxacin, and amikacin (subplots 1 to 4) and their related resistance genes found among the CNPA according to the literature. Vertical red dashed lines mark the EUCAST 2019 resistance breakpoints.

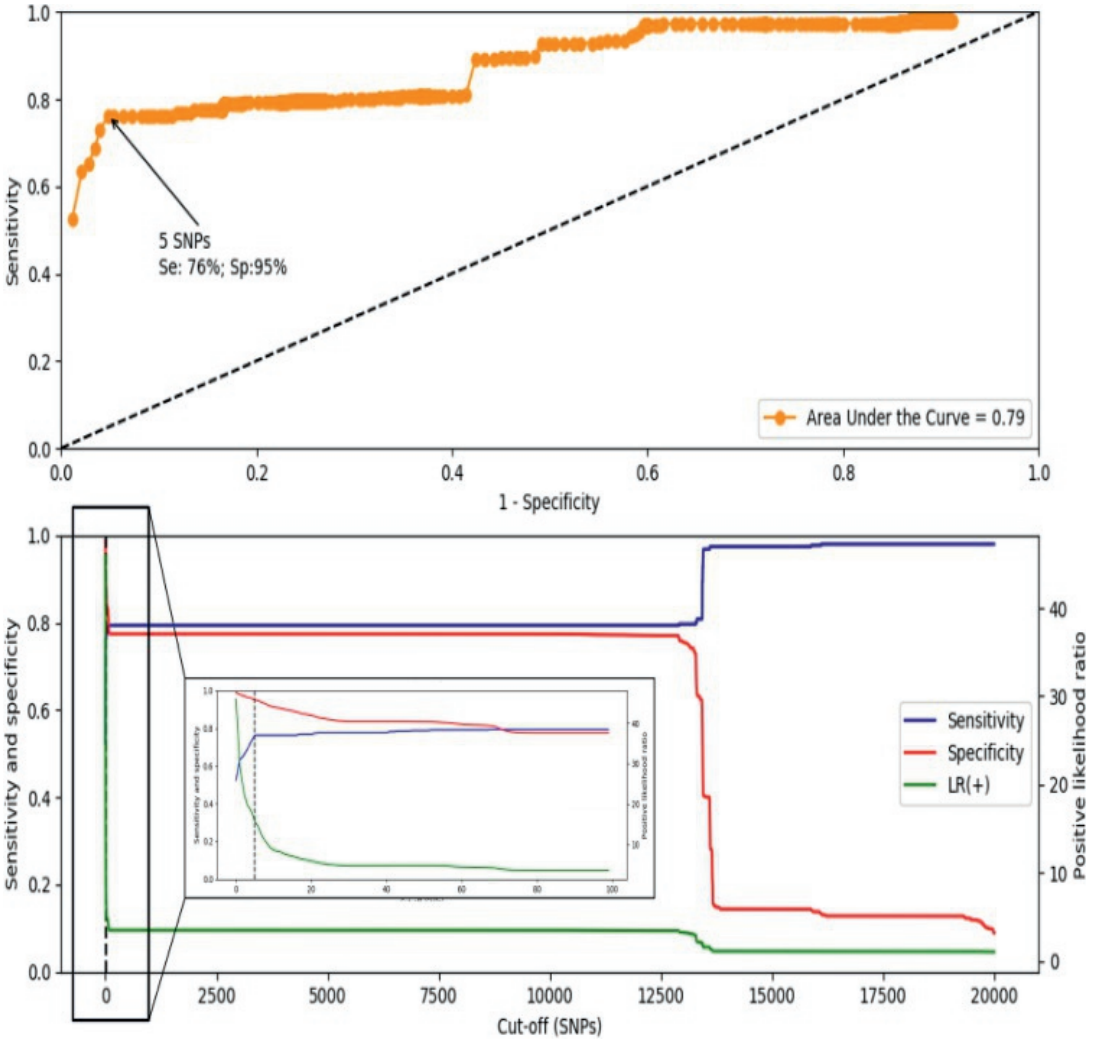

Figure 3. (Top image) Receiving operator characteristics curve. The area under the curve value is represented at the right corner of the image. (Bottom image) Cumulative distribution analysis showing the effects of variations in the cutoff SNP values on sensitivity (blue line), specificity (red line), and the positive likelihood ratio (green line). A vertical dashed black line shows the threshold corresponding to 5 SNP. A zoomed image of the first 100 cutoffs is displayed inside a box.

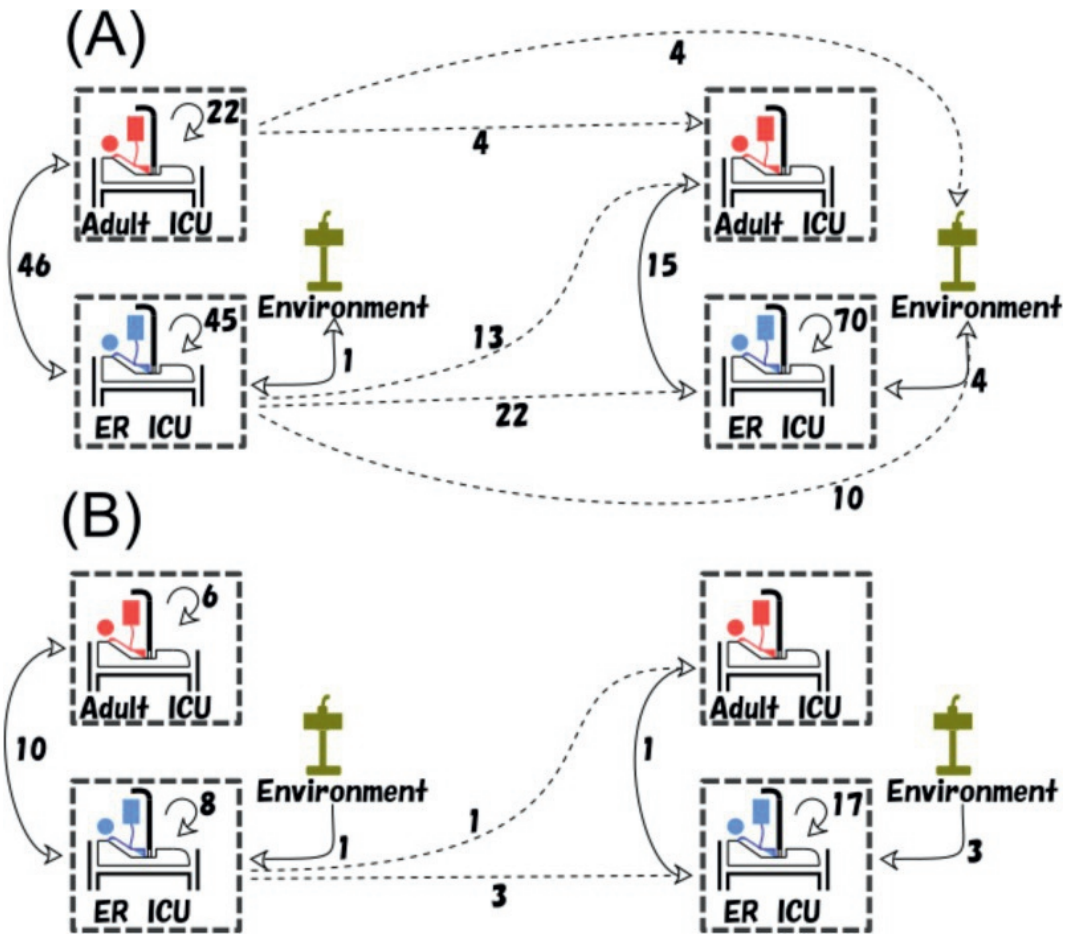

Figure 4. (A) Potential transmission events (PTEs) and (B) acquisition events (AEVs) from known sources during the study period. The boxes consisting of dashed lines represent the two intensive care units, during the preintervention and postintervention phases (panels on the left and right, respectively). Environmental sources of CNPA are depicted by green-colored sinks. Dashed lines represent transmissions (both PTEs and AEVs) between the two study phases; note that such transmissions have been registered as belonging to the postintervention phase.

Using the 5 SNP threshold, the PTEs occurred in the ICU setting at a rate of 27.7/100 admissions and 38.0/100 admissions during the Pre- and Post-intervention phases, respectively. However, the rate of AEVs was much lower, at 9.2/100 admissions in the Pre-intervention phase and 11.8/100 admissions in the Post-intervention phase. The majority of AEVs were from known sources. However, a sizable minority of AEVs (38% and 42% in the respective phases of the study) were from an unknown source.

Figure 4 displays the number of PTEs and AEVs from known sources during the study period. Most transmission events (PTEs and AEVs) occurred between patients within each of the two ICU's, but such transmissions were also noted to occur between the two ICU wards. Environmental sources were mostly linked to transmissions in the ER-ICU, although we registered four PTEs linking Adult-ICU patients admitted during the pre-intervention phase to CNPA positive environmental samples cultured during the post-intervention phase, indicating long term circulation of these strains in the ICU. Using either of the two approaches, we did not find a statistically significant difference between the PTE or AEV rates in the pre- versus the post-intervention period (p-values > 0.05). However, the observed number of AEVs in the Adult ICU dropped from six to zero in the pre- versus the post-intervention periods. In contrast, AEVs increased from eight to 17 in the ER-ICU at the same time. Similar contrary trends were observed while tracing PTEs.

Finally, the calculated Hill Numbers for the collections of CPNA isolated in the pre- and post-intervention phases, increased (^{0}D = 20 and 29, ^{1}D = ≈10 and ≈20, ^{2}D = ≈7 and ≈17), indicating that the genotypic diversity among the CNPA isolates cultured during the post-intervention phase had increased compared to the genotypic diversity of the collection before the intervention.

DISCUSSION

In this study, carbapenem non-susceptible *Pseudomonas aeruginosa* (CNPA) strains were found to be endemic in the ICUs of the Dr. Cipto Mangunkusumo General Hospital in Jakarta, Indonesia. CNPA clones are present in the ICU environment and are regularly being transmitted to and from patients and their immediate environment. We detected four dominant *P. aeruginosa* clones (ST235, ST357, ST446 and ST823), which have previously been shown to have spread worldwide and carry a repertoire of antimicrobial resistance related genes, from carbapenemases (such as bla_{IMP} or bla_{VIM}) to defective outer membrane porins (see below). We also traced CNPA transmissions using whole-genome sequencing (WGS). To do so we distinguished isogenic CNPA strains using a new methodology based on the differences in the SNP profiles of their core genomes and on patients from whom they were cultured. An optimal SNP threshold – below which strains are considered isogenic -, was calculated and then applied to trace transmissions of CNPA in this setting: one approach identified potential transmission events based solely on isogenic

strains being cultured from different sites. In another approach we additionally used clinical data to infer CNPA acquisitions by patients during their ICU stay. The latter approach helped us focus on CNPA transmission routes relevant for nosocomial infection control.

We also assessed the impact of an infection control intervention that was implemented in both ICUs to reduce transmissions of multidrug resistant pathogens. We did observe a large shift in the distribution of CNPA clones, together with an increase in the genotypic diversity of the CNPA population. However, overall the rates of acquisition of CNPA strains by patients during their ICU stay did not change significantly. Interestingly and inexplicably, a reduced transmission rate in one ICU was accompanied by an increased transmission rate of CNPA in the other ICU. Since the study did not include comparator ICU's that were not intervened with, the observed changes might well be a reflection of the natural variation in the epidemiological dynamics of CNPA in such settings. Although both ICUs were under same management, the ICUs are in different buildings, have dedicated nursing staffs, and also differ in turnover of patients, which all might have confounded the effects of the intervention. Either prospective comparative study designs or quasi-experimental designs such as interrupted time series analysis would be needed to come to more definitive evaluations of the effect of hygienic interventions on the epidemiology of CNPA in intensive care units.

The four main sequence types described in the ICUs of this hospital in Jakarta belong to the so called *P. aeruginosa* high-risk international clones reported around the globe that are associated with MDR profiles: ST235 and ST357 have been extensively reported in several countries [26–29], while the other two main STs, ST823 and ST446, have more recently emerged. A recent study of MDR *P. aeruginosa* from Malaysia also found ST235, ST357 and ST446 in a hospital setting [30]. ST823 has already been described as a minor sequence type found in a multicentre study undertaken in the Gulf Cooperation Council states; more specifically, this clone was found among isolates from Qatar and the United Arab Emirates [31]. ST823 has also been found in India and was shown to contain an atypically long genomic island harbouring $bla_{\text{VIM-2}}$ [32]. ST446 has been isolated in small clusters or singletons in Spain, The Netherlands, Eastern-France and Belgium, showing MDR or carbapenemase-producing profiles [33–36]. None of the minor sequence types of CNPA in this study harbored genes coding for carbapenemases, including the ST244 strains, which in a previous study from West and Central Africa have been linked to the production of $bla_{\text{VIM-2}}$ carbapenemase [37]. One of the possible consequences of the intervention we cannot readily explain is the substitution of ST235 by the ST357 as the dominant clone. We argue that this substitution may have been the consequence of the intervention, cleaning up environmental sites and limiting transmission initially but not being able to maintain hygienic vigilance over time [14]. Alternatively, the waxing and waning of different CNPA sequence types over time may be the rule rather than the exception in ICUs, and this natural trend may not have

been much influenced by the infection control intervention applied in our ICU setting. In addition, we did not observe significant changes in the number and composition of AMR determinants in both sequence types, which augurs against changes in antibiotic pressure as a direct cause of the replacement of the CNPA strains. Other hypotheses we contemplated are changes in the virulence patterns, as in the study of Bricio-Moreno *et al*. (2017) [38], or a different susceptibility to certain disinfectants such as chlorhexidine, as chlorhexidine bathing and mouth wash was part of the intervention.

We wanted to see the full repertoire of AMR genes present in our collection, because *P. aeruginosa* is well known for its capacity to expand its resistome, especially in hospital settings, with the ICU as an important hotspot [39]. In a recent study determining the resistomes of 672 *P. aeruginosa* strains, Jaillard et al. identified 147 loci associated with antimicrobial resistance, including associations between AMR-markers and antibiotics not described before [40]. Around 40% of the AMR determinants described in our collection overlapped with those described by Jaillard *et al.* These differences might be explained by the huge diversity in the *P. aeruginosa* genome, i.e.: over 30 different aminoglyoside-resistance markers were present, plus mutations in other AMR determinants such as *mexZ* or *fusA1* could contribute to the global aminoglycoside resistance of the strains [25]. Predictably, over two thirds of the genotypically unique strains (87/130, 66.9%) in our CNPA collection carried a carbapenem-degrading enzyme. Back in 2015, Potron *et al*. [41], published a review focusing on the AMR mechanisms and epidemiology of mutltidrug resistant *Acinetobacter baumannii* and *P. aeruginosa* which featured several tables including all known carbapenemases, including those found in our study. Interestingly, while $bla_{\text{GES-5}}$, $bla_{\text{IMP1-7-43}}$, and $bla_{\text{VIM-2}}$ had been previoulsy reported in Asian countries, including Japan, China, India and Malysia, $bla_{\text{VIM-8}}$ had only been previoulsy reported in Colombia, South America [42]. To our knowledge, this study is the first one to report this specific $bla_{\text{VIM-8}}$ type outside of South America.

Another well known mechanism of non-susceptibility to carbapenems is a defective OprD, an outer membrane porin in *P. aeruginosa* [43]. We found several amino acid substitutions and insertion and/or deletion events in the tertiary structure of the porin protein OprD, but we could not associate them to specific resistance phenotypes. All these point mutations, have been previously described [44–46]. Specific point mutations (i.e.: early stop codons) and frame shifts may be involved in the loss of the OprD porin, and, therefore, affect carbapenem susceptibility [47,48].

The spread of MDR Gram-negative bacteria carrying carbapenem resistance genes, such as those described above, has been largely influenced by several factors in a local and global scale, including pathogen and host characteristics, antibiotic prescription practices, and public health policies [49]. Furthermore, there is increasing evidence relating antibiotic consumption to the rise

of AMR. A recent retrospective study in 153 tertiary hospitals in China significantly correlated the use of carbapenems to the rate of carbapenem-resistant Gram-negative bacteria including *P. aeruginosa* [50]. To address this issue, antimicrobial stewardship programs (ASP) aimed to optimize the use of broad-spectrum antibiotics, have been set up in different forms and contexts [51]. According to a recent meta-analysis, ASP outcomes translate to less broad-spectrum antibiotics consumed and less infections by MDR microorganisms, among others benefits [52]. As an example, an ASP to restrict the use of carbapenems was implemented in the ICU of a Saudi Arabian hospital, effectively reducing the prevalence of MDR strains among the *P. aeruginosa* isolates [53].

Until a few years ago hospitals from high-income countries relied on techniques, such as PFGE, to classify nosocomial pathogens into genetically closely related groups called genotypes, such that their epidemiology could be ascertained. However, WGS provides maximum discriminatory power and is deemed able to unequivocally assign identity to them [54]. Two WGS-typing approaches have emerged: Multi locus sequence type based on whole/core-genomes (wg/cgMLST) or analysis of Single Nucleotide Polymorphisms across the whole/core-genome (wg/cgSNP)[55,56]. Our typing method, based on cgSNPs and clinical data, depends on the quality of each of these data sources. The calculated optimal SNPs threshold value depends to some degree on the genotypic composition of the collection of isolates under analysis. We have shown that the diversity of bacterial population may influence this cut-off value, although it remains fairly similar to the 4 SNPs threshold established in other studies [11,57]. Thus, there may not be a single optimal SNP cut-off value to distinguish isogenic strains across different collections of *P. aeruginosa*. However, an optimal SNP cutoff value can be derived for each collection using the new method we describe in this report.

We would like to point out some other limitations in our study design, the main being the relatively small number of environmental isolates included. Environmental samples were only taken once during each of the two phases of the study. Thus, much more frequent environmental sampling is needed, as was done for the patients themselves, to better detect the niches and transmission routes of CNPA in the innate environment of the ICUs. Similarly, healthcare personnel was sampled only twice, which may not be a sufficiently sensitive method to exclude their potential role as (intermediate) reservoirs, sources or vectors of CNPA. Another issue is that we did not target carbapenem-susceptible *P. aeruginosa* (CSPA) which would have allowed a deeper understanding of the role of resistance genes in the epidemiology of *P. aeruginosa* in this healthcare setting and would have further clarified the development of the resistome of this important nosocomial pathogen. Finally, all our calculations were made with a couple of custom Python scripts, and some manual counting. This makes the process not fully automated and, as it

is now, not fully scalable. Further steps to improve the scalability and reproducibility of this methodology are necessary.

CONCLUSIONS

Using whole genome sequencing in combination with clinical data, we were able to closely track and trace the endemic spread of isogenic carbapenem non-susceptible strains of *Pseudomonas aeruginosa* over a three year period in ICUs of a single tertiary care hospital in Indonesia, a large tropical middle-income country. We observed significant changes in the clonal composition of CNPA and provide insight into the dynamics of transmission of these strains over time, but could not directly ascribe these changes to the infection control interventions applied. Additionally, we detected the presence of high-risk international clones of multidrug resistant *P. aeruginosa* in Indonesia and present their resistomes.

DECLARATION

Acknowledgements

We thank the staff of the Department of Anesthesia and Intensive Care, Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia for their commitment and cooperation.

Funding

1. **Yulia Rosa Saharman** is an awardee of the DIKTI-NESO Scholarship by The Directorate General of Higher Education of Indonesia Ministry of Research, Technology and Higher Education of the Republic of Indonesia, and Department of Medical Microbiology and Infectious Diseases, Erasmus MC in Rotterdam, The Netherlands.
2. **Andreu Coello Pelegrin** and **Mattia Palmieri** received funding from the European Union's Horizon 2020 research and innovation programme "New Diagnostics for Infectious Diseases" (ND4ID) under the Marie Skłodowska-Curie grant agreement N° 675412.

Conflict of interest

ACP, MP, CM, AG and AvB are employees of bioMérieux, a company developing, marketing and selling tests in the infectious disease domain. The company had no influence on the design and execution of the clinical study neither did the company influence the choice of the diagnostic tools used during the clinical study. The opinions expressed in the manuscript are the author's which do not necessarily reflect company policies.

Ethics and Regulatory Considerations

Ethical Approval

The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the research on 17th September 2012, No: 561/PT02.FK/ETIK/2012, (No: 757/UN2.F1/ETIK/X/2014).

Material Transfer Agreement (MTA)

A Material Transfer Agreement (MTA) was reviewed and approved by the Director of National Institute Research and Development, Ministry of Health (No: LB.02.01/I.9.4/8500/2013).

Trial registration.

The study was registered at www.trialregister.nl (No:5541). Candidate number: 23527, NTR number: NTR5541, Date registered NTR: 22nd December 2015

Consent for publication

Informed consent was documented by the use of a written consent form approved by the Ethics Committee Faculty of Medicine Universitas Indonesia/Dr. Cipto Mangunkusumo General Hospital and signed and dated by the subjects/guardians and by the person who conducted the informed consent discussion and two witnesses. The signature confirmed the consent was based on information that had been understood.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at

https://doi.org/10.1128/mBio.02384-19.

Table S1:

Detailed antibiotic susceptibility testing performed by Vitek 2 of the 237 isolates. Categories of isolates are indicated as follows: Y (yes), isolates that appear in Results in the article; N (no), isolates excluded from description because of genotype overrepresentation. Download Table S1, XLSX file, 0.02 MB.

Table S2:

Whole-genome sequencing quality control parameters. *, average nucleotide identity analysis: reference *Pseudomonas aeruginosa* PAO1 (GenBank accession no. NC_002516.2).

Download Table S2, XLSX file, 0.04 MB.

Table S3:

Detailed results of multilocus sequence typing of the 237 isolates. Download Table S3,DOC file, 0.05 MB.

Table S4:

Direct and intrinsic mutational resistomes associated with antimicrobial resistance. “Direct mut. Resistome” indicates missense variations leading to amino acid point mutations in genes directly associated with antimicrobial resistance to β-lactams, aminoglycosides, quinolones, and/or polymyxins. “Intrinsic mut. resistome” indicates intrinsic genes (mainly RND efflux pumps determinants and regulators) with amino acid point mutations. Blank boxes signify that no missense variation was found. Download Table S4, XLSX file, 0.10 MB.

Table S5:

Detailed information regarding the clinical data of the samples and patients included in the study. Download Table S5, XLSX file, 0.1 MB.

Table S6:

Table showing the effect of the genotypic composition of the input samples in the cutoff calculation. “original” indicates the current composition of the collection of carbapenem-nonsusceptible *P. aeruginosa* isolates. Each “NOT” row represents removal of all isolates of one of the main sequence types described. Download Table S6, DOCX file, 0.01 MB.

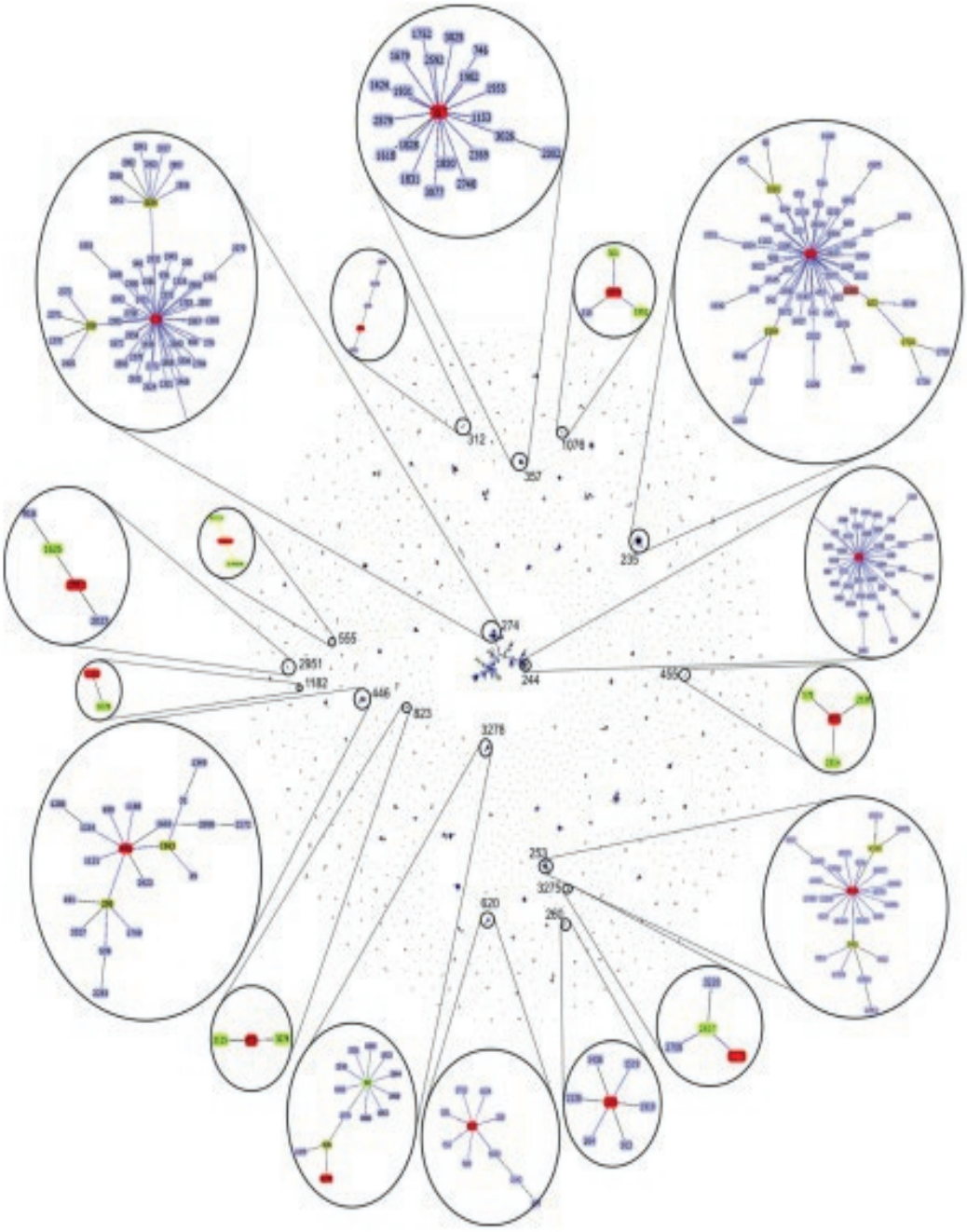

Figure S1: goeBURST of the 3321 sequence types listed in the *Pseudomonas aeruginosa* PubMLST database (August 2019). Blue points represent sequence types (STs); lines connect single-locus variants (SLVs). Red boxes within the circles are the STs found in this study, light green boxes represent the clonal complex group founder, while dark green boxes the sub-group founder. ST1189 and ST3277 are singletons.

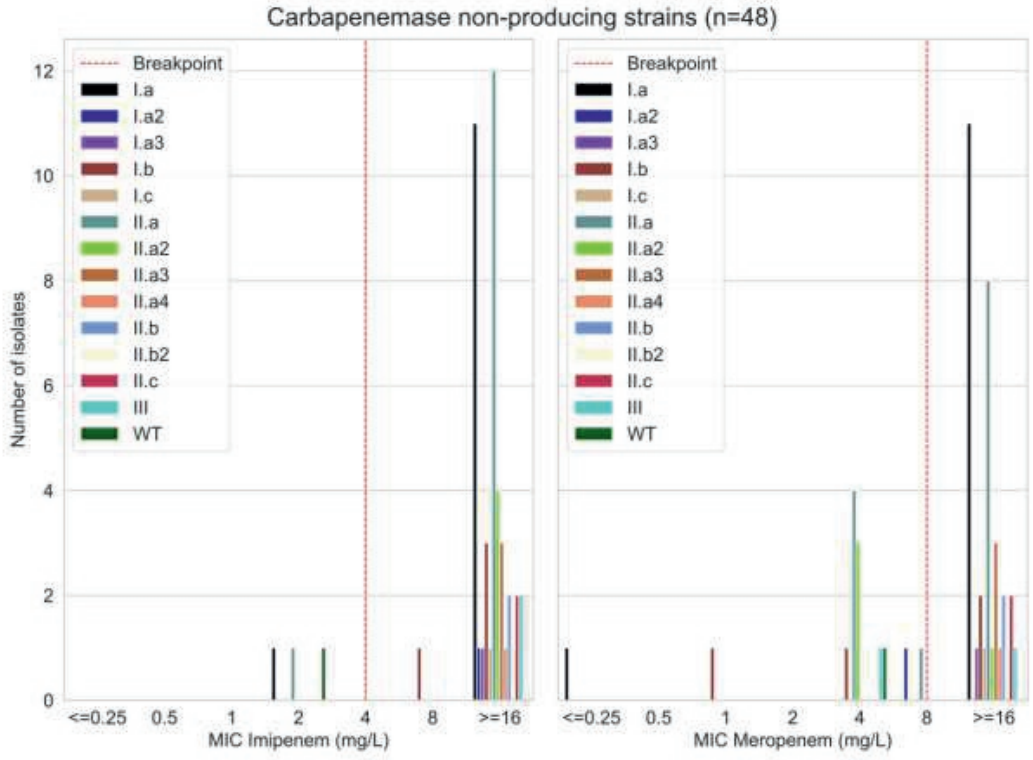

Figure S2: OprD types among the carbapenemase non-producing strains. Three main OprD types and 11 other sub-types were observed. The point mutation pattern is described below, note that sub-types *I.a*, *I.a2*, and *I.a3* additionally carried the $_{372}$(VDSSSS-YAGL-)$_{383}$ indel in the C-terminal part of OprD, firstly described by Epp, *et al*. [40].*I.a* (S_{57}E, S_{59}R, V_{127}L, E_{185}Q, P_{186}G, V_{189}T, E_{202}Q, I_{210}A, E_{230}K, S_{240}T, N_{262}T, T_{276}A, A_{281}G, K_{296}Q, Q_{301}E, R_{310}E, A_{315}G, L_{347}M, S_{403}A, Q_{424}E);

- *I.a2* (V_{127}L, E_{185}Q, P_{186}G, V_{189}T, E_{202}Q, I_{210}A, E_{230}K, S_{240}T, N_{262}T, T_{276}A, A_{281}G, K_{296}Q, Q_{301}E, R_{310}E, A_{315}G, L_{347}M, S_{403}A, Q_{424}E);
- *I.a3* (S_{57}E, S_{59}R, V_{127}L, E_{185}Q, P_{186}G, V_{189}T, E_{202}Q, I_{210}A, E_{230}K, S_{240}T, N_{262}T, T_{276}A, A_{281}G, K_{296}Q, Q_{301}E, R_{310}E, A_{315}G, **V_{352}L**, S_{403}A, Q_{424}E).
- *I.b* (D_{43}N, S_{57}E, S_{59}R, V_{127}L, E_{185}Q, P_{186}G, V_{189}T, E_{202}Q, I_{210}A, E_{230}K, S_{240}T, N_{262}T, **A_{267}S**, A281G, K296Q, Q301E, R310G, **V_{359}L**);
- *I.c* (S_{57}E, S_{59}R, V_{127}L);
- *II.a* T_{103}S, K_{115}T, F_{170}L, E_{185}Q, P_{186}G, V_{189}T, R_{310}E, A_{315}G, G_{425}A
- *II.a2* T_{103}S, K_{115}T, F_{170}L, E_{185}Q, P_{186}G, V_{189}T, R_{310}E, G_{425}A
- *II.a3* T_{103}S, K_{115}T, F_{170}L, E_{185}Q, P_{186}G, V_{189}T, R_{310}E, A_{315}G, **C_{420}R**
- *II.a4* T_{103}S, K_{115}T, F_{170}L, E_{185}Q, P_{186}G, V_{189}T, R_{310}E, A_{315}G
- *II.b* T_{103}S, K_{115}T, F_{170}L
- *II.b2* D_{43}N, T_{103}S, K_{115}T, F_{170}L
- *II.c* T_{103}S, K_{115}T, F_{170}L, E_{185}Q, P_{186}G, V_{189}T, R_{310}E, A_{315}G, Y_{350}STOP
- *III* W_{417}STOP

CHAPTER 10

10

SUMMARIZING DISCUSSION / NEDERLANDSE SAMMENVATING / DISKUSI DAN RINGKASAN (BAHASA INDONESIA)

Summarizing Discussion

Carbapenem-non-susceptible Gram-negative bacteria constitute one of the biggest threats to global health, especially to lower-middle income countries and one of these countries is Indonesia. Indonesia is the fourth populous country in the world, but at the time this study started only few data on such resistant strains were reported from Indonesia. Intensive care units (ICUs) are high-risk areas for transmission of antimicrobial-resistant bacteria. Carbapenem use is particularly high in this setting. However, in many cases, patients are treated with antimicrobial agents without proper clinical microbiological diagnosis (1), thus, the attending doctors tend not to really know what the causes of the patients' illnesses are, and habitually prescribe broad spectrum antibiotics. To improve this situation, first we need proper local microbiological data and surveillance, they are key to any infection control program.

In this thesis, we have studied the molecular epidemiology and resistance mechanisms of carbapenem-non-susceptible *Acinetobacter baumannii, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in two ICUs in the National Referral Hospital in Jakarta, Indonesia. After an initial baseline epidemiological survey, an intervention was designed using a set of inexpensive measures that were deemed feasible to implement in a low-resource ICU. In this chapter, the main findings are summarized and discussed on the basis of objectives of the study. Furthermore, suggestions for further research on the different topics are presented.

Objective 1 of the study: *To obtain a baseline insight into the epidemiology and the phenotypic and genetic characteristics of carbapenem-non-susceptible strains of Acinetobacter baumannii, Pseudomonas aeruginosa and Klebsiella pneumoniae in a low-resource ICU setting.*

In total, we included 412 patients in this baseline period, 188 were admitted to the adult ICU and 224 to the emergency room (ER-)ICU. Overall, 38% patients had a positive culture with carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex (CNAB), and 14% patients carried a carbapenem-non-susceptible *K. pneumoniae* (CNKP) at a certain moment during their ICU stay. Carbapenem-non-susceptible *P. aeruginosa* (CNPA) were isolated from 12% patients. (2-4)

Screening cultures taken on the day of ICU admission revealed that 17% patients already carried CNAB, 5% carried a CNKP strain, and 4% patients carried a CNPA strain.(2-4) With this data, we can conclude that some patients already carry carbapenem-non-susceptible strains when entering the ICU. Such patients may carry more than one species of carbapenem-non-susceptible bacteria. (see Figure 1) This should increase our awareness that patients who are admitted into ICU are potential sources of such strains.

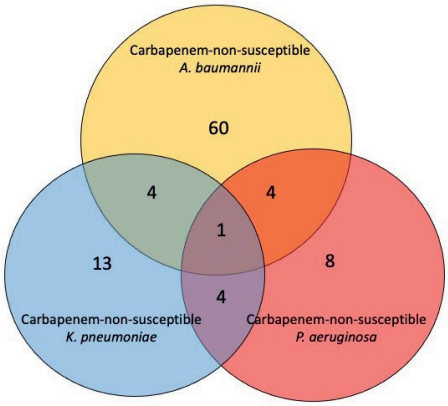

Figure 1. Venn Diagram of carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex, *K. pneumoniae* and *P. aeruginosa* on admission

During ICU stay 26% patients who were initially culture-negative acquired carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex, 9% a carbapenem-non-susceptible *K. pneumoniae* and 8% patients acquired a carbapenem-non-susceptible *P. aeruginosa.* The acquisition rate was, thus, the highest for carbapenem-non-susceptible *A. baumanni-calcoaceticus* complex (43 per 1000 patient-days at risk), followed by carbapenem-non-susceptible *K. pneumoniae* (25 per 1000 patient-days at risk) and carbapenem-non-susceptible *P. aeruginosa* (18/1000 patient- days at risk).(2-4) Again, quite a few patients acquired more than one species of carbapenem-non-susceptible strains.(see Figure 2)

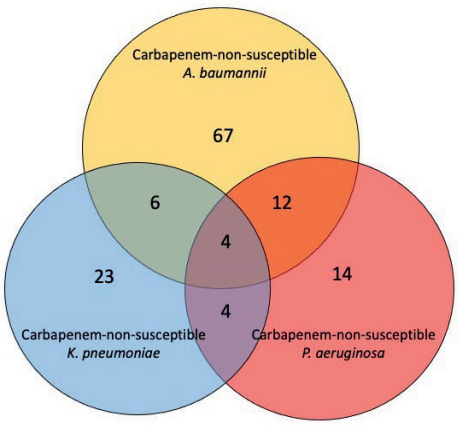

Figure 2. Venn Diagram of carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex, *K. pneumoniae* and *P. aeruginosa* during ICU stay.

Acquisition of carbapenem-non-susceptible strains occurred quite rapidly after admission to the ICU, within one-week half of the patients that acquired such strain had already done so, and by day 12-14 more than 80% of patients had acquired their resistant strain. This indicates that the acquisition risk was always present and was always high in these ICUs. Indeed, the most consistent risk factor for the acquisition of the strains was the use of carbapenem antibiotics.

Acquisition of carbapenem-non-susceptible strains of these three species was associated with poor outcomes, patients' lengths of stay were much longer and the observed mortality rates were higher (>40%) among those with carbapenem-non-susceptible strains compared to patients not acquiring such strains (< 30%). Patients that acquired multiple carbapenem-non-susceptible strains belonging to two or more species had an even higher mortality rate (50%), where as those patients that acquired only a single such strain had a lower mortality rate (39.4%). In contrast, patients that acquired carbapenem-susceptible strains of *K. pneumoniae* or of *P. aeruginosa* had low mortality rates (<20%), compatible with the lower severity of their illnesses and with less exposure to mechanical ventilation and to carbapenem antibiotics. Thus, patients can also acquire susceptible strains of *K. pneumoniae* and of *P. aeruginosa* during their ICU stay. To the contrary, virtually all strains of *A. baumannii* circulating in the ICU were carbapenem-non-susceptible.

The innate environment may contain niches where antibiotic resistant strains survive and, even multiply.(5, 6) Environmental screening was done twice in the baseline phase of this study; from a total of 400 samples we uncovered six carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex isolates (cultured from table, bed rails, sinks, and tap water).(2) In addition, one water sample (taken from a suction connector) yielded a *K. pneumoniae* strain that was carbapenem-non-susceptible. Remarkably, 16 strains of carbapenem-non-susceptible *P. aeruginosa* were isolated from the environment (3, 4) This finding was very important, since it clearly implicated the environment as a potentially significant source of carbapenem-non-susceptible strains, clearly it should be a target for interventions that aim to reduce the acquisition rate of resistant species, especially of resistant *P. aeruginosa* and *A. baumannii.*

Health care workers were also screened as a potential source of transmission. Only a single isolate from a healthcare worker (throat) was CNAB,(2) none of 24 *K. pneumoniae* isolates cultured from HCWs (out of 167 screened) were found to be carbapenem-non-susceptible,(4) and none of 25 *P. aeruginosa* strains isolated from HCWs was carbapenem-non-susceptible, suggesting that HCW do not play a significant role in the epidemiology of such strains in the ICU setting.(3) However, a clear limitation of this study was that HCW screening was done only one time, and only throat and rectal samples were cultured (not hands). We could, therefore, not properly assess the contribution of HCWs to the acquisition of such strains by ICU patients. Future studies entailing large scale and repeated sampling of HCWs are needed to properly define their role in the epidemiology of CNAB, CNKP and CNPA in ICU's.

High-level resistance of carbapenem is primarily mediated by carbapenemases. These enzymes occur mainly among Gram-negative pathogens such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, and may be intrinsic or mediated by transferable carbapenemase-encoding genes.(7)

A. baumannii has a native chromosomal oxacillinase (OXA-51 and its variants) that usually undergoes low-level expression, but it can potentially confer carbapenem resistance when upregulated following upstream insertion of the insertion element IS*Aba1* or IS*Aba9*. However, it is *A. baumannii*'s ability to acquire carbapenemases, specifically the Ambler class B metallo-β-lactamases and class D oxacillinases and recently class A carbapenemases as well, that has resulted in the widespread development of carbapenem-resistant *A. baumannii*.(7, 8) In our study, we found the $bla_{\text{OXA-23-like}}$ gene in 92% isolates including isolates from patients, the environment and from the healthcare worker. The $bla_{\text{OXA-24-like}}$ gene was detected in a single isolate. Coexistence of OXA-23 with other oxacillinases and carbapenemases was found: OXA-23/OXA-58 (1 isolate), and OXA-23/NDM-1 (4 isolates). The $bla_{\text{OXA-23-like}}$ gene was always demonstrated in combination with the IS*Aba1* insertion element upstream to the OXA-23 β-lactamase. The intrinsic *A. baumannii-calcoaceticus* complex gene $bla_{\text{OXA-51-like}}$ was demonstrated in all isolates. In the subset of isolates that were subjected to whole-genome sequencing (WGS), the $bla_{\text{OXA-51- like}}$ gene involved was $bla_{\text{OXA-66}}$ in 13 isolates and $bla_{\text{OXA-68}}$ in one isolate.(2)

Carbapenem-non-susceptibility among *K. pneumoniae* may be due to production of Ambler class A β-lactamases (e.g. KPC), class B metallo- β-lactamases (MBLs, e.g. VIM, IMP, NDM) or class D oxacillinases (e.g. OXA-48 like enzymes).(4, 8) The phenotypic detection test of CNKP indicated that 96% isolates produced an MBL. PCRs of carbapenemase genes demonstrated the presence of the bla_{NDM} gene in all carbapenem-non-susceptible isolates, including isolates from patients and the one from the environment. None of isolates was positive for either the bla_{KPC} or $bla_{\text{OXA-48}}$ gene.

Non-susceptibility to carbapenem antibiotics in *P. aeruginosa* is usually due to a combination of mechanisms, including β-lactamase production, increased efflux pump activity and outer membrane modifications or to production of a carbapenemase as a single potent resistance mechanism; VIM, IMP and GES-5 carbapenemases are most commonly found around the world.(3, 9) Using resistome analysis, CNPA was explained by the presence of various carbapenemase-encoding genes ($bla_{\text{GES-5}}$, $bla_{\text{VIM-2-8}}$, and $bla_{\text{IMP-1-7-43}}$) and by mutations within the porin OprD.(6) Phenotypic testing showed that 57% isolates in our study produced a MBL. PCR demonstrated the presence of bla_{VIM} in 30% and bla_{IMP} in 19%, including isolates from patients and the environment. None of the isolates were positive for bla_{NDM}. The presence of non-MBL $bla_{\text{GES-5}}$ was detected in 20% isolates.(3)

In summary, we found that carbapenem-non-susceptibility of these three species was largely based on the production of β-lactamases, but these three species did not share the same genes coding for the carbapenemases.

Hospital-associated infections (HAI) outbreaks caused by pathogenic strains have dramatic repercussions. In order to tackle this problem, infection-control teams use a broad range of typing techniques to help trace bacterial sources, to define the mode of dissemination of pathogenic clones and, ultimately, to hinder or preferably stop microbial spread. Most routine medical microbiology laboratories across the world currently use molecular biological techniques for this purpose. For the last decades Pulsed-Field Gel Electrophoresis (PFGE; based on the fragmentation of the bacterial genome by a specific enzyme) has been one of the most commonly used techniques in diagnostic laboratories across the world. In addition, Multi-locus variable number of tandem repeats (VNTR) analysis (MLVA), multi-locus sequence typing (MLST, based on polymorphisms in a limited number of housekeeping genes) are employed. During the last few years, whole-genome sequencing (WGS) has become a preferred tool for the molecular epidemiological surveillance of infectious diseases.(6) In the beginning of the study, we also used Raman spectroscopy as a rapid phenotypic bacterial typing method.(10) We were able to apply this technique to differentiate strains of *Klebsiella pneumoniae* and *Acinetobacter baumannii,* but the technique could not be applied to *P. aeruginosa* due to interference by pyocins that are intrinsic to this species.

Typing with Raman spectroscopy revealed five major clusters of CNAB, the largest cluster (designated CIPTO-31) consisted of isolates obtained from patients (screening and clinical specimens) and isolates from the environment throughout the study period. MLST, performed for a subset of 14 isolates, revealed the presence of multiple sequence types (STs), which corresponded closely to the Raman spectroscopy clustering. Four previously identified STs (ST195, ST208, ST218, and ST642) as well as several new STs, and a new allele for the *gpi* gene were found in this study.(2)

Raman spectroscopy analysis also revealed the presence of multiple types of CNKP. There were three major clusters, the largest cluster (CIPTOKPN24) consisted of isolates obtained from patients (screening and clinical specimens) and were present in both ICUs throughout the study period, whereas other clones seemed to wax and wane with time.(4) A total of 97 clinical and 1 environmental isolate were further analyzed using MLVA genotyping, identifying 30 different genotypes. Clustering of strains by Raman spectroscopy into three dominant groups was concordant with clustering by MLVA, e.g. the 20 Raman CIPTOKPN24 strains all belonged to a single MLVA clonal complex. Likewise, the four Raman CIPTOKPN30 strains belonged to a single MLVA clonal complex as did CIPTOKPN27 isolates.(4)

MLST revealed four major clusters of CNPA (ST235, ST823, ST446 and ST357) as well as several new sequence types. By MLVA, five major clusters were distinguished, two belonging to ST235

and the others corresponding to ST823, ST446 and ST357. Most isolates belonged to ST235 (patient and environmental isolates), of which 22 isolates harbored bla_{IMP}, 24 isolates harbored bla_{GES-5} but no isolates contained bla_{VIM}. All ST823 isolates harbored $bla_{VIM.}$

We further evaluated five molecular typing techniques using an epidemiologically well-characterized set of CNPA. They were Multi-Locus Variable Number of Tandem Repeat (VNTR) Analysis (MLVA), *in-silico* seven-loci multi-locus sequence typing (MLST), core and whole genome MLST (cg/wgMLST), and core Single Nucleotide Polymorphism (SNP) analysis (cgSNP). Our findings show that the three latter techniques (cgMLST, wgMLST and cgSNP) provide the highest level of resolution allowing detailed epidemiological analysis of local outbreaks and international dissemination. MLVA is a suitable alternative for accurate typing of *P. aeruginosa*, useful in settings where the transition towards WGS is currently not feasible.

Thus, the overall impression is that carbapenem-non-susceptible strains of the three species targeted by our studies show endemicity of a few clones that are circulating among ICU patients and the ICU environment.

Objective 2 of the study: *To develop an intervention - feasible to be applied in a low-resource ICU setting - that may significantly reduce the risk of acquisition and infection by carbapenem-non-susceptible A. baumannii, P. aeruginosa and K. pneumoniae.*

A large percentage of HAI are preventable through effective infection prevention and control (IPC) measures.(11) After phase 1, we introduced a multimodal bundle of IPC interventions that initially consisted of the following measures (12) and see chapter 8:

1. A multifaceted hand hygiene improvement program.
   We based the hand hygiene programs on the WHO's Five Moments for Hand Hygiene guidelines and tools. The hand hygiene improvement program included education with pre- and post-questionnaires testing of knowledge and attitudes, performance feedback and reminders, interviews, and role models as described before.(12)
2. A single round of an environmental disinfection campaign involving the whole environment of both ICUs using 1:100 sodium hypochlorite solution. This disinfectant solution was applied to walls, floors, doors, beds (mattresses and bed rails), sinks, overbed tables, infusion and suction pumps and stands, monitors and ventilators including connecting lines, surrounding counter tops including the adjacent cleaning service room. In addition, all curtains between beds were exchanged for clean ones.
3. Routine environmental disinfection was introduced with 1:100 sodium hypochlorite solution that included the floors, beds, and immediate surrounding of the patients. This was done twice daily. In case of visible dirt, this was first removed with a brush and water,

before the application of the sodium hypochlorite solution. The intensive procedure as described above was repeated every two weeks in this phase. The curtains between beds were refreshed every 1-2 months or immediately after visible soiling.

4. Enforced antibiotic stewardship (including daily evaluation of all antibiotic prescriptions on weekdays).
5. All patients found positive for one or more carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex, *K. pneumoniae*, or *P. aeruginosa* were cohorted in one dedicated corner of the ICU. HCWs donned mask, gown, and gloves when approaching and providing care for cohorted patients.
6. Daily total body-washing with cloths soaked in a chlorhexidine gluconate 2% solution. These cloths were prepared and pre-packaged individually in sealed plastic bags by the hospital pharmacy.
7. Introduced a 2% chlorhexidine gluconate solution for decontamination of the oropharynx. Bottles containing this solution were also prepared and provided by the hospital and used per patient. Oral decontamination was performed 4 time daily.

Objective 3 of the study: *To apply and determine the efficacy of the intervention (developed as specified above/ under 2) in a low-resource ICU setting.*

We measured HH knowledge and HH compliance before (at baseline) and directly after a multifaceted improvement program (post-intervention) and performed a re-evaluation three years later. The multifaceted improvement program included education, feedback, reminders, interviews and the use of role models. There was a statistically significant improvement in the median overall HH knowledge score at post-intervention.

The overall HH compliance was 27% at baseline and significantly improved to 77% post-intervention. For all five HH moments, the compliance of nurses and physicians separately improved significantly from the baseline phase to the post intervention phase, except for 'moment 3' (after body fluid exposure), for which baseline rates were already high. Most of the compliance rates were significantly lower in both groups of healthcare workers upon follow-up three years later, they essentially had fallen back to pre-intervention levels. Overall, the HH compliance of the nurses was significantly better than the physicians' compliance.(12) Thus, maintaining high levels of HH compliance requires continuous monitoring and regular interventions.

We evaluated the effect on the acquisition of carbapenem-non-susceptible *Acinetobacter baumannii-calcoaceticus* complex (CNAB), *Klebsiella pneumoniae* (CNKP), and *Pseudomonas aeruginosa* (CNPA). Using a quasi-experimental before-and-after design study, for all three

species taken together there was a significant step change, from phase 1 to phase 3 in the rate of acquisition of carbapenem-non-susceptible strains. This significant decrease in the overall acquisition rate of carbapenem-non-susceptible strains of the three species was mainly caused by a decrease in the acquisition of carbapenem-non-susceptible *A. baumannii-calcoaceticus* complex and, to a lesser extent, *K. pneumoniae.* Interestingly, the acquisition rate of *P. aeruginosa* was little affected by the multimodal intervention. Within each of the two phases there was no major downward or upward trend observed in the rate of acquisition of resistant strains for any of the three species separately nor for the three species taken together, although the risk of acquisition was increasing slightly in phase 1.(see chapter 8)

Using WGS in combination with clinical data, we were able to closely track and trace the endemic spread of isogenic carbapenem-non-susceptible strains of *Pseudomonas aeruginosa* over a 3-year period in the ICUs. We found that the number of CNPA transmissions and acquisitions by patients was highly variable over time but that, overall, the rates were indeed not significantly reduced by the intervention. Environmental sources were involved in these transmissions and acquisitions. Four high-risk international CNPA clones (ST235, ST823, ST357, and ST446) dominated, but the distribution of these clones changed significantly after the intervention was implemented.(6) Thus, the multimodal intervention may have altered the clonal composition of endemic carbapenem-non-susceptible *P. aeruginosa* but it did not eradicate the sources or niches nor affected the transmission of such strains in the ICU environment.

We conclude that a multimodal intervention aiming to prevent acquisition of resistant strains of important ICU pathogens is essentially feasible and may be quite effective in ICUs in lower-middle income countries. However, even multimodal interventions may not be equally effective for all species harbouring antibiotic-resistant strains.

RECOMMENDATION FOR FURTHER RESEARCH

Our study was conducted in two ICUs in a single center, the national reference hospital in Jakarta, Indonesia. Since Indonesia is a large country and the fourth most populous in the world, the data presented in this thesis do not represent the whole of Indonesia.

In the future, a national surveillance program for carbapenem-non-susceptible Gram-negative bacilli should be established, including all provinces of Indonesia, with collection of epidemiological data, phenotypic and genotypic resistance mechanisms, and analysis of clonal relatedness. An alert system should be set up for the recognition of “high-risk” clones or unnoticed transmission routes. For example, our finding that some patients already carried carbapenem-non-susceptible isolates on admission to the ICU, could be suggestive for

transmission between hospitals when patients are transferred or referred, or even suggestive for the existence of reservoirs in the community. These possible sources and routes should be further explored.

We found a limited variety of carbapenemase genes in each species, CNAB only had $bla_{\text{oxa-23}}$ and $bla_{\text{oxa-51}}$ genes, CNKP only carried bla_{NDM} and CNPA carried bla_{VIM}, $bla_{\text{GES-5}}$ and $bla_{\text{IMP..}}$ Do we also find the same carbapenemase-coding genes among ICU isolate of these three species in the other provinces in Indonesia? That still questionable. A surveillance system would generate data on this.

We concluded that a multimodal intervention aiming to prevent acquisition of resistant strains of important ICU pathogens is feasible and may be quite effective in ICUs in lower-middle income countries, but not for CNPA. Environmental cleaning seemed to be a very important part of the intervention with CNPA. *Pseudomonas aeruginosa* is a unique pathogen, this organism can survive over a long-term period and live in moist niches. There are two likely transmission pathways for CNPA, i.e. via exposure to untreated (waste)water and via contaminated sinks/taps in the hospital. This hypothesis needs to be explored by multicentre collaborative research efforts.

We found in our study, that the use of antibiotics, especially carbapenems, in ICUs is a major independent risk factor for the acquisition of carbapenem-non-susceptible strains. We, therefore, suggest that antimicrobial stewardship should be introduced in ICU daily practice and that its effects on the epidemiology of antimicrobial resistance be studied in this setting.

Also, the application of the intervention was not monitored except for the hand hygiene compliance. Future studies should closely monitor environmental cleaning (by observation of cleaning practices, and by much more environmental culturing), monitor the use of chlorhexidine body and oral decontamination) and last, but not least, it should be monitored whether patients with positive cultures are actually cohorted in the designated space or room. This should all be registered in future studies, essentially providing accurate data on the implementation of multimodal interventions.

Finally, when building new ICU facilities, even in low-resource settings, one should pay much more attention to avoid creating typical environmental niches for ICU pathogens and provide more structural barriers to the survival and spread of micro-organisms in general.

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Nederlandse Samenvatting

Resistentie van bacteriën voor antimicrobiële middelen is wereldwijd een toenemend probleem. Met name de carbapenem-ongevoelige Gram-negatieve bacteriën vormen één van de grootste bedreigingen voor de wereldgezondheid, vooral in lage- en middeninkomenslanden. Indonesië is volgens de Wereldbank een land met een laag-midden inkomen, het is tevens het vierde dichtstbevolkte land ter wereld. Op het moment dat deze studie begon waren er zeer beperkt gegevens bekend over het voorkomen van dergelijke resistente stammen in Indonesië.

In het algemeen is er een verhoogd risico op verspreiding van resistente bacteriën in ziekenhuizen, en dan vooral op intensieve zorgafdelingen ("intensive care units", IC's). Resistente bacteriën worden daar meer dan elders uitgeselecteerd door frequent gebruik van breedspectrum antibiotica, zoals de carbapenems; in lager-middeninkomenslanden gebeurt dat vaak zonder een klinische microbiologische diagnose (1). Om deze situatie te verbeteren zijn goede lokale microbiologische data nodig. In **hoofdstuk 2** van dit proefschrift wordt een overzicht van de literatuur gepresenteerd die gaat over op de IC verkregen infecties in dergelijke landen. Uit de beperkt beschikbare gegevens bleek dat het spectrum van pathogenen op IC's in laag-middeninkomenslanden anders is dan in hoge-inkomenslanden: resistente *Acinetobacter baumannii*, *Pseudomonas aeruginosa* en *Klebsiella pneumoniae* worden vaker gevonden.

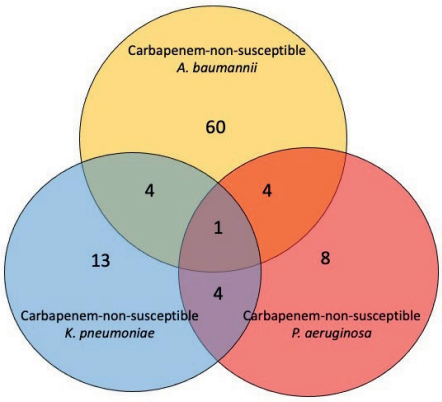
In dit proefschrift hebben we de moleculaire epidemiologie en resistentiemechanismen van de drie genoemde soorten bacteriën bestudeerd op twee IC's in het National Referral Hospital in Jakarta, Indonesië: carbapenem-ongevoelige *A. baumannii*, *P. aeruginosa* en *K. pneumoniae*. Allereerst werd de uitgangssituatie onderzocht. Daarna werd een pakket van haalbare maatregelen ontworpen om het vóórkomen en de verspreiding van deze bacteriën op ICs te verminderen en dit pakket werd geïmplementeerd als interventie. Hieronder worden de belangrijkste bevindingen samengevat en besproken aan de hand van de doelstellingen van het onderzoek.

Doelstelling 1: *Een inzicht verkrijgen in de epidemiologie en de fenotypische en genetische kenmerken van carbapenem-ongevoelige stammen van A. baumannii, P. aeruginosa en K. pneumoniae in twee IC's in Jakarta voorafgaand aan de interventie.*

Er werden 412 patiënten geïncludeerd in deze eerste fase van het onderzoek, 188 patiënten werden opgenomen op de IC voor volwassenen (IC-V) en 224 op de IC van de spoedeisende hulp afdeling (IC-SEH). In totaal had 38% van de patiënten een positieve kweek met een carbapenem-ongevoelige *A. baumannii* (CNAB), en 14% van de patiënten had op een bepaald moment tijdens hun IC-verblijf een carbapenem-ongevoelige *K. pneumoniae* (CNKP) bij zich.

Carbapenem-ongevoelige *P. aeruginosa* (CNPA) werden geïsoleerd bij 12% van de patiënten (**hoofdstuk 3, 4, 5**) (2-4).

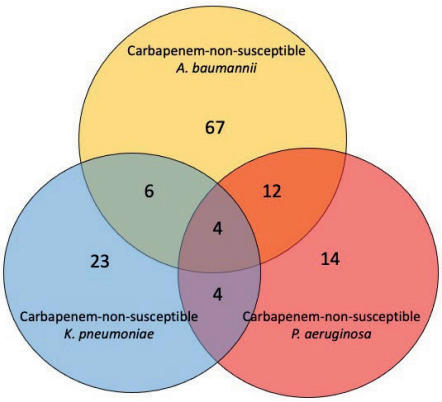
Uit screeningskweken die op de dag van opname op de IC werden afgenomen, bleek dat 17% van de patiënten al drager was van een CNAB, 5% van een CNKP en 4% van een CNPA (2-4). Patiënten bleken meer dan één soort carbapenem-ongevoelige bacteriën bij zich te kunnen dragen (Figuur 1). Deze bevinding zou ons bewustzijn moeten vergroten dat patiënten die op de IC in Jakarta worden opgenomen, bronnen van dergelijke resistente bacteriën kunnen zijn.



Figuur 1. Venn Diagram van carbapenem-ongevoelige *A. baumannii*, *K. pneumoniae* en *P. aeruginosa* bij opname

Tijdens hun IC-verblijf kreeg 26% van de patiënten die aanvankelijk bij opname negatieve screeningskweken hadden, een CNAB, 9% een CNKP en 8% een CNPA. De acquisitiefrequentie was dus het hoogst voor CNAB (43 per 1000 patiëntdagen), gevolgd door CNKP (25 per 1000 patiëntdagen) en CNPA (18 per 1000 patiëntdagen) (2-4). Nogal wat patiënten werden met meer dan één soort carbapenem-ongevoelige bacteriën besmet (Figuur 2).

Acquisitie van carbapenem-ongevoelige bacteriestammen trad vrij snel op na opname op de IC, binnen een week had de helft van de patiënten die een dergelijke stam kregen deze al opgedaan, en op dag 12-14 had meer dan 80% van de patiënten hun resistente stam. Dit geeft aan dat het acquisitierisico altijd aanwezig en altijd hoog was op deze IC's. De meest consistente risicofactor voor het verkrijgen van dergelijke stammen was inderdaad het gebruik van carbapenem antibiotica.



Figuur 2. Venn Diagram van carbapenem-ongevoelige *A. baumannii*, *K. pneumoniae* en *P. aeruginosa* verkregen tijdens verblijf op de IC

Het tijdens het IC verblijf verkrijgen van één van deze drie carbapenem-ongevoelige bacteriën was negatief geassocieerd met het resultaat van de IC-zorg. De verblijfsduur van de patiënten was veel langer en de waargenomen sterftecijfers waren hoger (> 40%) onder degenen met carbapenem-ongevoelige bacteriën in vergelijking met patiënten die dergelijke stammen niet verworven (<30%). Patiënten die twee of drie van de carbapenem-ongevoelige bestudeerde bacteriën verkregen hadden een nog hoger sterftecijfer (50%), terwijl patiënten die slechts met één zo'n resistente stam besmet werden een lager sterftecijfer hadden (39,4%). Patiënten die carbapenem-gevoelige stammen van *K. pneumoniae* of *P. aeruginosa* verkregen hadden juist een lage mortaliteit (<20%), een uitkomst dat paste bij de lagere ernst van hun ziekte en met minder blootstelling aan mechanische beademing en carbapenem antibiotica. Patiënten kunnen dus ook gevoelige stammen van *K. pneumoniae* en *P. aeruginosa* verkrijgen tijdens hun IC-verblijf. De *A. baumannii* stammen die op de IC circuleerden waren echter vrijwel allemaal ongevoelig voor carbapenem antibiotica.

De ziekenhuisomgeving kan niches bevatten waar antibioticaresistente bacteriën overleven en zelfs vermenigvuldigen (5, 6). Het kweken van de omgeving werd tweemaal uitgevoerd in de eerste fase van deze studie; uit in totaal 400 kweken hebben we zes CNAB-stammen geïsoleerd (uit monsters afgenomen van tafels, bedhekken, gootstenen en kraanwater) (2). Bovendien leverde één watermonster (genomen uit een afzuigaansluiting) een *K. pneumoniae*-stam op die niet gevoelig was voor de carbapenems. Opmerkelijk was dat 16 carbapenem-ongevoelige stammen van *P. aeruginosa* uit de omgeving werden geïsoleerd (3, 4). Deze bevindingen zijn belangrijk, omdat het duidelijk maakt dat de IC-omgeving een potentieel belangrijke bron is voor carbapenem-ongevoelige stammen; de IC-omgeving zou dus een doel moeten zijn voor interventies die gericht zijn op het verminderen van de acquisitie van resistente bacteriën, met name van resistente *P. aeruginosa* en *A. baumannii*.

Gezondheidspersoneel werd ook gescreend als mogelijke bron. Slechts één isolaat van een gezondheidsmedewerker (keel) bleek een CNAB (2), géén van de 24 *K. pneumoniae*-isolaten gekweekt uit 167 personeelsleden van de IC bleek ongevoelig voor carbapenems te zijn (4), en geen van de 25 gevonden *P. aeruginosa* stammen was ongevoelig voor carbapenem antibiotica, wat suggereert dat gezondheidsmedewerkers geen significante rol spelen in de epidemiologie van dergelijke stammen op de IC (3). Een beperking van deze studie was echter dat het kweken van personeel slechts één keer werd uitgevoerd en dat alleen keel- en rectumkweken werden geanalyseerd (geen handen). We konden daarom de bijdrage van personeel aan de epidemiologie van dergelijke stammen op de IC's niet goed inschatten. Toekomstige studies met uitgebreidere en herhaalde bemonstering van gezondheidsmedewerkers zijn nodig om hun rol in de epidemiologie van CNAB, CNKP en CNPA in IC's beter te definiëren.

Resistentie tegen carbapenem antibiotica wordt voornamelijk gemedieerd door carbapenemasen. Deze enzymen komen met name voor bij Gram-negatieve pathogenen zoals *K. pneumoniae*, *P. aeruginosa* en *A. baumannii*, en kunnen intrinsieke eigenschap van de bacterie zijn. Anderszins kunnen dergelijke enzymen worden verkregen door overdracht van carbapenemase-coderende genen van resistente stammen naar gevoelige soortgenoten(7).

A. baumannii heeft een chromosomaal oxacillinase (OXA-51 en zijn varianten) die gewoonlijk een lage expressie heeft, c.q. niet leidt tot klinisch relevante resistentie; maar deze genen kunnen carbapenem-resistentie leveren wanneer stroomopwaarts het insertie-element IS*Aba1* of IS*Aba9* aanwezig is. Het is echter het vermogen van *A. baumannii* om carbapenemasen te verwerven, met name de Ambler klasse B metallo-β-lactamasen (MBL, bijv. NDM, VIM, IMP) en klasse D oxacillinasen en recent ook klasse A carbapenemasen, dat heeft geresulteerd in het wijdverspreid opduiken van carbapenem-resistente *A. baumannii* (7, 8). In onze studie vonden we het $bla_{\text{OXA-23-like}}$ gen in 92% van de geïsoleerde stammen, dit is inclusief de isolaten van patiënten, de omgeving en van een gezondheidsmedewerker. Het $bla_{\text{OXA-24-like}}$ gen werd gedetecteerd in één stam. Het metallo-β-lactamase $bla_{\text{NDM-1}}$ werd bij 4 $bla_{\text{OXA-23-like}}$-positieve isolaten aangetoond. Het intrinsieke *A. baumannii-calcoaceticus*-complex gen $bla_{\text{OXA-51-like}}$ werd in alle isolaten aangetoond. In een subset van isolaten die werden geanalyseerd met "whole genome sequencing" (WGS) bleek het om het $bla_{\text{OXA-66}}$ gen te gaan in 13 isolaten en $bla_{\text{OXA-68}}$ in één isolaat (2).

Ook bij *K. pneumoniae* kan carbapenem-ongevoeligheid veroorzaakt worden door de productie van Ambler klasse A β-lactamases (bijv. KPC), klasse B MBL of klasse D oxacillinases (bijv. OXA-48) (4, 8). De fenotypische detectietest van CNKP gaf aan dat 96% van de isolaten een MBL produceerden. PCR's van carbapenemase-genen toonden de aanwezigheid van bla_{NDM} aan in alle carbapenem-ongevoelige isolaten, inclusief isolaten van patiënten en die uit de omgeving. Geen van de isolaten was positief voor het bla_{KPC} or $bla_{\text{OXA-48}}$ gen.

De carbapenem-ongevoeligheid bij *P. aeruginosa* is meestal te wijten aan een combinatie van mechanismen, waaronder de productie van β-lactamase, een activiteit van hun effluxpompen, en aan veranderingen van het buitenmembraan van deze bacteriën, óf het is te wijten aan de productie van een carbapenemase als één enkel krachtig resistentiemechanisme: VIM-, IMP- en GES-5-carbapenemasen worden wereldwijd het meest aangetroffen (3, 9). Met behulp van resistoomanalyse kon de carbapenem-ongevoeligheid bij de CNPA verklaard worden door de aanwezigheid van verschillende carbapenemase-coderende genen (*bla*$_{\text{GES-5}}$, *bla*$_{\text{VIM-2-8}}$, en *bla*$_{\text{IMP-1-7-43}}$) en door mutaties in de porine OprD (**hoofdstuk 9**) (6). *bla*$_{\text{VIM}}$ kwam het meest frequent voor (30% van de isolaten) (3).

Samenvattend vonden we dat de ongevoeligheid voor carbapenems van deze drie bacteriën grotendeels gebaseerd was op de productie van β-lactamasen die carbapenem antibiotica kunnen afbreken, maar deze drie bacteriesoorten hadden niet dezelfde carbapenemase genen.

Uitbraken van ziekenhuis- of zorg-gerelateerde infecties, zeker met resistente bacteriën, hebben dramatische gevolgen. Het is daarom belangrijk om bacteriële bronnen op te sporen en de verspreidingsroute van pathogene klonen te bepalen, zodat uiteindelijk de verspreiding van de pathogenen kan worden tegengegaan en bij voorkeur gestopt. Een breed scala aan typeringstechnieken is hiervoor beschikbaar. De meeste medische microbiologische laboratoria in de hele wereld gebruiken momenteel moleculair-biologische technieken voor dit doel. “Pulsed-Field Gel Electrophoresis” (PFGE; gebaseerd op de fragmentatie van het bacteriële genoom door een specifiek enzym) is de laatste decennia en wereldwijd een van de meest gebruikte technieken. Deze techniek is lang beschouwd als “gouden standaard”, maar heeft ook diverse beperkingen. Naast PFGE worden tegenwoordig “Multi-locus Variable Number of Tandem Repeats” (VNTR) analyse (MLVA) en de multilocus sequentietypering (MLST, gebaseerd op polymorfismen in een beperkt aantal huishoudgenen) gebruikt. In de afgelopen jaren is “whole genomen sequencing” (WGS) opgekomen als instrument voor de moleculaire epidemiologische surveillance van pathogenen (6). In het begin van onze studie hebben we Raman-spectroscopie gebruikt, een snelle en goedkope fenotypische bacteriële typeringsmethode (10). We waren in staat om deze techniek toe te passen om stammen van *K. pneumoniae* en *A. baumannii* te analyseren, maar de techniek kon niet worden toegepast op *P. aeruginosa* vanwege de pyocyanines die deze bacteriësoort produceren.

De analyse met Raman-spectroscopie bracht vijf grote clusters van CNAB aan het licht. Het grootste cluster (aangeduid als CIPTO-31) bestond uit isolaten verkregen van patiënten (van screeningskweken en klinische kweken) en isolaten uit de omgeving. MLST, uitgevoerd voor een subset van 14 isolaten, onthulde de aanwezigheid van meerdere sequentietypen (ST's), die nauw overeenkwamen met de Raman-spectroscopieclustering. Vier eerder geïdentificeerde ST's

(ST195, ST208, ST218 en ST642) evenals verschillende nieuwe ST's werden in deze studie gevonden (2).

Raman-spectroscopieanalyse onthulde ook de aanwezigheid van meerdere typen CNKP. Er waren drie grote clusters, het grootste cluster (CIPTOKPN24) bestond uit isolaten verkregen van patiënten (van screeningskweken en klinische kweken) en waren aanwezig op beide IC's gedurende de onderzoeksperiode, terwijl andere klonen leken te komen en gaan over de tijd (4). Een totaal van 97 klinische en 1 isolaat uit de IC-omgeving werden verder geanalyseerd met behulp van MLVA-genotypering, waarbij 30 verschillende genotypen werden geïdentificeerd. De clustering van isolaten door Raman-spectroscopie in drie dominante groepen kwam overeen met clustering door MLVA (4).

Ook de CNPA isolaten werden op meerdere manieren geanalyseerd. Met MLST werden vier belangrijke clusters van CNPA geïdentificeerd (ST235, ST823, ST446 en ST357) evenals verschillende nieuwe sequentietypen. Met MLVA werden vijf grote clusters onderscheiden, twee behorend tot ST235 en de andere overeenkomend met ST823, ST446 en ST357. De meeste isolaten behoorden tot ST235 (patiënten- en omgevingsisolaten), waarvan 22 isolaten bla_{IMP} bevatten en 24 isolaten $bla_{\text{GES-5}}$. Er waren geen ST235 isolaten met een bla_{VIM} gen. Daarentegen was een bla_{VIM} gen wel aanwezig in alle ST823-isolaten.

Vervolgens evalueerden we vijf moleculaire typeringstechnieken voor de CNPA: MLVA, MLST met zeven loci, “core genome” en “whole genome” MLST (cg / wgMLST) en “core genome Single Nucleotide Polymorphism” (cgSNP) analyse (**hoofdstuk 6**). Onze bevindingen tonen aan dat de drie laatste technieken (cgMLST, wgMLST en cgSNP) het hoogste resolutieniveau bieden, waardoor gedetailleerde epidemiologische analyse van lokale uitbraken en van eventuele internationale verspreiding mogelijk is. MLVA is een geschikt alternatief voor het typeren van *P. aeruginosa* daar waar de overgang naar WGS momenteel niet haalbaar is.

De algemene conclusie van deze analyses is dat de drie carbapenem-ongevoelige bacteriësoorten waar ons onderzoek op gericht was, endemisch voorkomen op de twee IC’s in Jakarta, waarbij slechts enkele persisterende klonen meer frequent worden gevonden bij patiënten en in de omgeving.

Doelstelling 2: *Een interventie ontwikkelen - haalbaar voor toepassing op een IC met weinig middelen - die het risico op het verkrijgen van een carbapenem-ongevoelige* A. baumannii*,* P. aeruginosa *en* K. pneumoniae *aanzienlijk zou kunnen verminderen.*

Zorggerelateerde infecties en verspreiding van pathogenen die deze veroorzaken kan worden voorkomen door effectieve infectiepreventiemaatregelen (11). Na de studieperiode waarin de uitgangssituatie werd bestudeerd, introduceerden wij een multimodale bundel van

infectiepreventiemaatregelen die aanvankelijk bestond uit de volgende onderdelen (**hoofdstuk 8**) (12):

8. Een verbeterprogramma voor handhygiëne.
   Dit verbeterprogramma werd gebaseerd op de handhygiënerichtlijnen en -hulpmiddelen van de Wereldgezondheidsorganisatie ("World Health Organization", WHO). Het programma omvatte onderwijs met vragenlijsten, het testen van kennis en attitudes, prestatiefeedback, geheugensteuntjes, interviews en het trainen van rolmodellen (12).
9. Een enkele ronde van schoonmaak en desinfectie waarbij de hele omgeving van beide IC's met een 1:100 natriumhypochlorietoplossing werd gedesinfecteerd. Deze desinfecterende oplossing werd aangebracht op wanden, vloeren, deuren, bedden (matrassen en bedhekken), wasbakken, bedtafels, infuus- en afzuigpompen en palen, beeldschermen en ventilatoren inclusief aansluitleidingen. Ook de aangrenzende spoelruimte/bijkeuken werd hierin meegenomen. Bovendien werden alle gordijnen tussen de bedden vervangen door schone.
10. Routinematige omgevingsdesinfectie met 1:100 natriumhypochlorietoplossing werd geïntroduceerd voor de vloeren, bedden en directe omgeving van de patiënten. Dit werd tweemaal daags gedaan. Bij zichtbaar vuil werd dit eerst verwijderd met een borstel en water, voordat de natriumhypochlorietoplossing werd aangebracht. De intensieve procedure zoals beschreven bij 2. werd bovendien elke twee weken herhaald. De gordijnen tussen de bedden werden elke 1-2 maanden verschoond of onmiddellijk na zichtbare vervuiling.
11. Versterkt "antibiotic stewardship" (inclusief dagelijkse evaluatie van alle antibioticavoorschriften van opgenomen patiënten op weekdagen).
12. Alle patiënten die positief werden bevonden voor één of meer carbapenem-ongevoelige bacteriën (CNAB, CNKP, CNPA) werden in een daarvoor aangewezen hoek van de IC-afdeling geplaatst, in een zogenaamd "cohort". Bij het verlenen van zorg aan deze patiënten trokken gezondheidsmedewerkers een isolatiejas, een mondneusmasker en handschoenen aan.
13. Dagelijkse wassen van het lichaam van alle patiënten met doekjes gedrenkt in een chloorhexidine gluconaatoplossing van 2%. Deze doekjes werden door de ziekenhuisapotheek zelf bereid en per stuk verpakt.
14. Introductie van een 2% chloorhexidinegluconaatoplossing voor decontaminatie van de orofarynx. Flessen met deze oplossing werden ook bereid en geleverd door de ziekenhuisapotheek en per patiënt gebruikt. Orale decontaminatie werd vier keer per dag uitgevoerd.

Doelstelling 3: *Het toepassen en bepalen van de effectiviteit van de interventie (ontwikkeld zoals hierboven bij doelstelling 2 gespecificeerd) op twee IC's met beperkte middelen in een ziekenhuis in Jakarta.*

Het verbeterprogramma voor handhygiëne staat beschreven in **hoofdstuk 7**. We hebben de kennis en compliantie van handhygiëne gemeten vóór en direct na het verbeterprogramma (de interventie) en voerden drie jaar later opnieuw een meting van de compliantie uit. Direct na het verbeterprogramma was er een statistisch significante verbetering in de mediane algehele kennisscore. De algehele handhygiënecompliantie was 27% bij aanvang en verbeterde significant tot 77% na de interventie. Voor alle vijf momenten van handhygiëne verbeterde de compliantie van verpleegkundigen en artsen afzonderlijk significant van de baselinefase tot de post-interventiefase, behalve voor 'moment 3' (na blootstelling aan lichaamsvloeistof), waarvoor de baselinecijfers al hoog waren. Bij follow-up drie jaar later bleken meeste compliantie percentages weer significant lager in beide groepen gezondheidswerkers, ze waren teruggevallen tot het niveau van vóór de interventie. Over het algemeen was de compliantie van de verpleegkundigen significant beter dan die van de artsen (12). Het behouden van een hoog niveau van naleving van handhygiëne vereist dus continue monitoring en regelmatige interventies.

In **hoofdstuk 8** beschrijven we een analyse van het effect van het hele pakket aan maatregelen op de acquisitie van carbapenem-ongevoelig *A. baumannii* (CNAB), *K. pneumoniae* (CNKP) en *P. aeruginosa* (CNPA) door patiënten op de twee IC's in Jakarta. De opzet was dus een quasi-experimentele voor-en-na-studie. De statistische analyse (met een regressiemodel) toonde aan dat er voor alle drie de bacteriën tezamen een significante stapsgewijze verandering, c.q. vermindering was in de kans op het verkrijgen van carbapenem-ongevoelige stammen, van fase 1 (de fase voor de interventie) naar fase 3 (de fase na de interventie). Deze significante afname in de totale acquisitie van carbapenem-ongevoelige stammen van de drie soorten werd voornamelijk veroorzaakt door een afname in de acquisitie van CNAB, en in mindere mate CNKP. Interessant genoeg werd de acquisitiesnelheid van CNPA weinig beïnvloed door de multimodale interventie. Binnen elk van de twee fasen was er géén sprake van een belangrijke opwaartse of neerwaartse trend in de mate van verwerving van resistente stammen voor een van de drie soorten afzonderlijk noch voor de drie soorten samen, hoewel het risico van acquisitie licht toenam in fase 1.

Een verdere analyse van het effect van de interventie op CNPA is beschreven in **hoofdstuk 9**. Met behulp van WGS in combinatie met klinische gegevens waren we in staat om de endemische verspreiding van isogene CNPA stammen gedurende de onderzoeksperiode van 3 jaar op de IC's nauwkeurig te volgen en te traceren. We ontdekten dat het aantal CNPA-

transmissies en acquisities door patiënten in de loop van de tijd zeer variabel was, maar dat de percentages over het algemeen inderdaad niet sterk werden verminderd door de interventie. Bij deze transmissies en acquisities waren bronnen in de omgeving betrokken. Vier CNPA-klonen, die bekend staan als intenationale hoog-risico klonen (ST235, ST823, ST357 en ST446) overheersten, maar de distributie van deze klonen veranderde aanzienlijk nadat de interventie was geïmplementeerd (6). De multimodale interventie kan dus de klonale samenstelling van endemische CNPA hebben veranderd, maar het heeft de omgevingsbronnen van CNPA niet gesaneerd, noch heeft het de overdracht van dergelijke stammen in de IC sterk belemmerd.

We concluderen dat een multimodale interventie gericht op het voorkomen en acquisitie van resistente stammen van belangrijke pathogenen haalbaar is en behoorlijk effectief kan zijn op IC's in landen met een lager-middeninkomensniveau. Echter, zelfs multimodale interventies zijn mogelijk niet even effectief voor alle antibioticaresistente stammen.

In **hoofdstuk 10** worden de belangrijkste resultaten besproken en in bredere context geplaatst. Tevens worden suggesties gedaan voor toekomstig onderzoek.

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Diskusi Dan Ringkasan (Bahasa Indonesia)

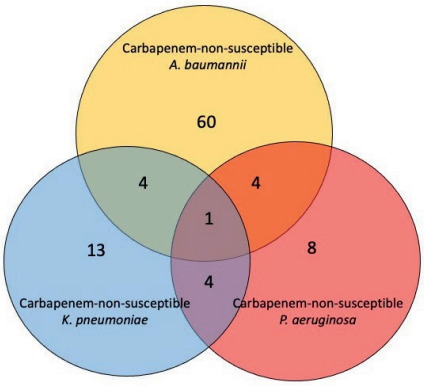
Bakteri gram negative yang resisten terhadap Karbapenem merupakan salah satu ancaman paling berbahaya pada kesehatan global, terutama di negara yang memiliki penghasilan yang menengah ke bawah dan salah satunya adalah Indonesia. Indonesia merupakan negara dengan jumlah penduduk keempat terbesar di dunia, tetapi pada saat studi ini dimulai, data mengenai strain resisten ini sangat sedikit yang sudah dilaporkan di Indonesia. Unit perawatan intensif (ICU) adalah area yang beresiko tinggi terjadinya transmisi bakteri yang resisten terhadap antimikroba. Penggunaan karbapenem sangat tinggi di ICU. Namun, dalam banyak kasus, pasien diobati dengan antimikroba tanpa diagnosis mikrobiologi klinik (1) demikian, dokter penanggung jawab cenderung tidak benar-benar mengetahui apa penyebab infeksi pada pasien, dan biasanya meresepkan antibiotik spektrum luas. Untuk memperbaiki situasi ini, pertama-tama kita membutuhkan data surveilans mikrobiologi lokal yang tepat, karena data ini merupakan kunci untuk setiap program pengendalian infeksi.

Dalam tesis ini, kami meneliti epidemiologi molekuler dan mekanisme resistensi *carbapenem-non-susceptible Acinetobacter baumannii, Klebsiella pneumoniae* dan *Pseudomonas aeruginosa* di dua ICU di Rumah Sakit Rujukan Nasional di Jakarta, Indonesia. Setelah survei epidemiologi awal dilakukan, intervensi dirancang menggunakan serangkaian tindakan yang murah dan dapat untuk diterapkan pada ICU dengan sumber daya yang terbatas atau dapat diterapkan di seluruh wilayah di Indonesia. Dalam bab ini, temuan utama dirangkum dan dibahas berdasarkan tujuan penelitian. Selain itu, saran untuk penelitian lebih lanjut tentang berbagai topik juga disajikan.

1. **Tujuan 1:** *Untuk mendapatkan data dasar tentang epidemiologi dan karakteristik fenotipik dan genetik dari strain carbapenem-non-susceptible Acinetobacter baumannii, Pseudomonas aeruginosa dan Klebsiella pneumoniae di ICU dengan sumber daya terbatas.*

Total sebanyak 412 inklusi pasien dalam periode ini, 188 pasien dirawat di ICU dewasa dan 224 pasien di ICU IGD (ER-ICU). Secara keseluruhan, 38% pasien memiliki kultur positif dengan *carbapenem-non-susceptible A. baumannii-calcoaceticus* complex (CNAB) dan 14% pasien positif dengan *carbapenem-non-susceptible K. pneumoniae* (CNKP) selama dirawat di ICU. *Carbapenem-non-susceptible P. aeruginosa* (CNPA) diisolasi dari 12% pasien. (2-4)

Kultur untuk skrining yang diambil pada hari pertama dirawat di ICU ditemukan bahwa 17% pasien sudah terkolonisasi dengan CNAB, 5% membawa strain CNKP, dan 4% pasien membawa strain CNPA. (2-4) Dengan data ini, kita dapat menyimpulkan bahwa beberapa pasien sudah membawa strain *carbapenem-non-susceptible* saat mulai rawat di ICU. Pasien tersebut dapat memiliki lebih dari satu spesies bakteri yang *carbapenem-non-susceptible*. (lihat Gambar 1)

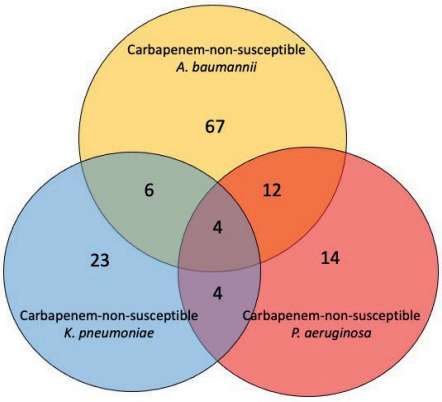


Gambar 1. Diagram Venn *carbapenem-non-susceptible A. baumannii-calcoaceticus* complex, *K. pneumoniae* dan *P. aeruginosa* pada saat admisi.

Hal ini harus meningkatkan kesadaran kita bahwa pasien yang dirawat di ICU adalah sumber penularan potensial dari strain tersebut.

Selama dirawat di ICU, 26% pasien yang awalnya kultur-negatif mendapatkan *carbapenem-non susceptible A. baumannii-calcoaceticus* complex, 9% carbapenem-non-susceptible *K. pneumoniae* dan 8% pasien positif *carbapenem-non-susceptible P. aeruginosa.* Tingkat akuisisi yang tertinggi adalah *A. baumanni-calcoaceticus* complex (43 per 1000 hari pasien berisiko), diikuti oleh *carbapenem-non-susceptible K. pneumoniae* (25 per 1000 hari pasien berisiko) dan *carbapenem-non-susceptible P. aeruginosa* (18/1000 hari pasien beresiko) (2-4) Sekali lagi, beberapa pasien memperoleh lebih dari satu spesies *carbapenem-non-susceptible* strain.(lihat Gambar 2)

Akuisisi *carbapenem-non-susceptible* strain terjadi secara cepat setelah pasien masuk ke ICU, dalam waktu satu minggu, setengah dari pasien mendapatkan strain tersebut, dan pada hari 12-14 lebih dari 80% pasien telah memperoleh starin resisten tersebut. Ini menunjukkan bahwa risiko akuisisi selalu ada dan selalu tinggi dalam kondisi ICU ini. Memang, faktor risiko yang paling konsisten untuk akuisisi strain resisten ini adalah penggunaan antibiotik karbapenem.



Gambar 2. Diagram Venn *carbapenem-non-susceptible A. baumannii-calcoaceticus* complex, *K. pneumoniae* dan *P. aeruginosa* selama dirawat di ICU.

Akuisisi strain *carbapenem-non-susceptible* dari ketiga spesies ini dikaitkan dengan luaran pasien yang buruk, lama tinggal pasien yang jauh lebih lama dan tingkat kematian yang lebih tinggi (>40%) di antara mereka dengan *carbapenem-non-susceptible* dibandingkan dengan pasien yang tidak memperoleh strain seperti itu (< 30%). Pasien yang terinfeksi dengan dua spesies atau lebih strain *carbapenem-non-susceptible* memiliki tingkat kematian yang lebih tinggi (50%), di mana pasien yang hanya terinfeksi oleh satu strain tersebut memiliki tingkat kematian yang lebih rendah (39,4%). Sebaliknya, pasien yang memperoleh *carbapenem-susceptible K. pneumoniae* atau *P. aeruginosa* memiliki tingkat kematian yang rendah (<20%), sesuai dengan tingkat keparahan penyakit mereka yang lebih rendah dan dengan paparan ventilasi mekanis dan antibiotik karbapenem yang lebih sedikit. Dengan demikian, pasien juga dapat memperoleh strain *K. pneumoniae* dan *P. aeruginosa* yang sensitif selama dirawat di ICU. Sebaliknya, hampir semua strain *A. baumannii yang* beredar di ICU adalah *carbapenem-non-susceptible*.

Lingkungan ICU dapat mengakibatkan kondisi strain resisten antibiotik bertahan dan, bahkan berkembang biak. (5, 6) Skrining lingkungan dilakukan dua kali dalam fase dasar penelitian ini; dari total 400 sampel, kami menemukan enam isolat *carbapenem-non-susceptible A. baumannii-calcoaceticus* complex (kultur dari meja, rel/pinggir tempat tidur, wastafel, dan air keran). (2) Selain itu, satu sampel air (diambil dari konektor *suction*) menghasilkan strain *carbapenem-non-susceptible K. pneumoniae.* Luar biasanya, 16 strain *carbapenem-non-susceptible* P. *aeruginosa* terisolasi dari lingkungan tersebut (3, 4) Temuan ini sangat penting, karena jelas implikasinya bahwa lingkungan dapat berpotensi menjadi sumber penularan dari strain *carbapenem-non-susceptible* signifikan, dan harus menjadi target intervensi yang bertujuan untuk menurunkan angka tingkat akuisisi spesies resisten tersebut, terutama *P. aeruginosa* dan *A. baumannii.*

Skrining terhadap petugas kesehatan juga dilakukan sebagai potensi sumber penularan. Hanya satu isolasi dari tenaga kesehatan (tenggorokan) adalah CNAB,(2)dari 24 isolat *K. pneumoniae* yang dikultur dari petugas kesehatan (dari 167 yang diskrining) tidak ada yang merupakan isolat *carbapenem-non-susceptible*,(4) strain P. *aeruginosa* yang diisolasi dari petugas kesehatan, tidak ada yang c *carbapenem-non-susceptible*. Hal ini menunjukkan bahwa HCW tidak memainkan peran penting dalam epidemiologi strain CNAB, CNKP and CNPA di ICU. (3) Namun, keterbatasan atau limitasi yang jelas dari penelitian ini adalah bahwa skrining HCW dilakukan hanya satu kali, dan hanya kultur dari swab tenggorok dan rektal (bukan dari tangan). Oleh karena itu, kita tidak bisa menilai dengan benar kontribusi HCW untuk akuisisi strain tersebut pada pasien ICU. Studi di masa depan yang berskala besar dan pengambilan sampel HCW berulang diperlukan untuk mendefinisikan peran mereka dengan benar dalam epidemiologi CNAB, CNKP dan CNPA di ICU.

Resistensi karbapenem yang tinggi dimediasi oleh enzim karbapenemase. Enzim ini muncul terutama pada bakteri Gram-negatif seperti *Klebsiella pneumoniae, Pseudomonas aeruginosa* dan *Acinetobacter baumannii*, dan mungkin intrinsik atau dimediasi oleh gen *Carbapenemase-encoding* yang dapat ditransfer. (7)

A. baumannii memiliki kromosom oksisilinase (OXA-51 dan variannya) yang biasanya diekspresikan secara rendah, tetapi berpotensi berubah menjadi karbapenem resisten ketika diupregulasi setelah *upstream insertion* elemen *Insertion* tersebut IS*Aba1* atau IS*Aba9*. Namun, itu adalah kemampuan *A. baumanni* untuk memperoleh enzim karbapenemase, khususnya kelas *Ambler B metallo-lactamases* dan *oxacillinases kelas D* dan baru-baru ini karbapenemase kelas A juga, yang mengakibatkan penyebaran luas *carbapenem-resistant A. baumannii*. (7, 8) Dalam penelitian ini, kami menemukan gen $bla_{\text{OXA-23-like}}$ pada 92% isolat termasuk isolat dari pasien, lingkungan dan dari petugas kesehatan. Gen $bla_{\text{OXA-23-like}}$ terdeteksi dalam satu isolat. Koeksistensi OXA-23 dengan oksisilinase dan karbapenemase lainnya ditemukan: OXA-23/OXA-58 (1 isolat), dan OXA-23/NDM-1 (4 isolat). Gen $bla_{\text{OXA-23-like}}$ selalu ditemukan berkombinasi dengan IS*Aba1 insertion element upstream* ke OXA-23 β-laktamase. Gen intrinsik *A. baumannii-calcoaceticus* complex $bla_{\text{OXA-51-like}}$ didapatkan pada semua isolat. Dalam beberapa isolat yang diidentifikasi dengan *whole-genome sequencing* (WGS), gen $bla_{\text{OXA-51- like}}$ yang terlibat adalah $bla_{\text{OXA-66}}$ pada 13 isolat dan $bla_{\text{OXA-68}}$ pada satu isolat. (2)

Resistensi terhadap karbapenem pada *K. pneumoniae* mungkin disebabkan oleh produksi enzim *Ambler kelas A β-lactamases* (misalnya KPC), kelas *B metallo- β-lactamases* (MBLs, misalnya VIM, IMP, NDM) atau *oxacillinases kelas D* (misalnya OXA-48 seperti enzim). (4, 8) Tes deteksi fenotipik CNKP menunjukkan bahwa 96% isolat menghasilkan enzim MBL. PCR gen karbapenemase menunjukkan adanya gen bla_{NDM} di semua *Carbapenem-non-susceptible* isolat,

termasuk isolat dari pasien dan lingkungan. Tidak ada satu isolat pun yang positif terhadap gen bla_{KPC} atau bla_{OXA-48}.

Resistensi terhadap antibiotik karbapenem pada *P. aeruginosa* biasanya disebabkan oleh kombinasi beberapa mekanisme resistensi, termasuk produksi *β-lactamase*, peningkatan aktivitas pompa efflux dan modifikasi membran luar atau produksi enzim karbapenemase sebagai mekanisme tunggal resistensi yang kuat; VIM, IMP dan GES-5 adalah enzim karbapenemase paling sering ditemukan di dunia. (3, 9) Dengan menggunakan analisis resistome, CNPA dijelaskan oleh adanya berbagai gen pengkode karbapenemase (bla_{GES-5}, $bla_{VIM-2-8}$,d an $bla_{IMP-1-7-43}$) dan dengan adanya mutasi porin oprD. (6) Pengujian fenotipik menunjukkan bahwa 57% isolat dalam penelitian kami menghasilkan enzim MBL. PCR menunjukkan adanya gen bla_{VIM} dalam 30% isolay dan bla_{IMP} dalam 19% isolat, termasuk isolat dari pasien dan lingkungan. Tidak ada isolat yang positif untuk bla_{NDM}. Kehadiran gen non-MBL bla_{GES-5} terdeteksi dalam 20% isolat. (3)

Simpulan, kami menemukan bahwa *carbapenem-non-susceptible* dari ketiga spesies ini sebagian besar berdasarkan pada produksi enzim β-laktamases, tetapi ketiga spesies ini tidak mengkode gen karbapenemase yang sama.

Hospital-associated infections (HAI) yang disebabkan oleh strain patogen memiliki dampak yang dramatis. Untuk mengatasi masalah ini, tim pengendalian infeksi menggunakan berbagai teknik *typing* untuk membantu melacak sumber bakteri, untuk menentukan model penyebaran klon patogen dan, pada akhirnya, untuk menghambat atau lebih menghentikan penyebaran mikroba. Sebagian besar laboratorium mikrobiologi klinik di seluruh dunia saat ini rutin menggunakan teknik biologi molekuler untuk tujuan ini. Selama beberapa dekade terakhir *Pulsed-Field Gel Electrophoresis* (PFGE; berdasarkan fragmentasi genom bakteri oleh enzim tertentu) telah menjadi salah satu teknik yang paling umum digunakan di laboratorium diagnostik di seluruh dunia. Selain itu, *multi-locus variable number of tandem repeats (VNTR) analysis (MLVA)*, *multi-locus sequence typing* (MLST, berdasarkan polimorfisme gen *housekeeping* dalam jumlah terbatas) juga digunakan. Selama beberapa tahun terakhir, *whole-genome sequencing* (WGS) telah menjadi metode yang disukai untuk surveilans epidemiologi molekuler penyakit menular. (6) Pada awal penelitian, kami juga menggunakan Raman spektroskopi sebagai metode cepat *typing* bakteri secara fenotipik. (10)*Klebsiella pneumoniae dan Acinetobacter baumannii,* tetapi teknik ini tidak dapat diterapkan pada *P. aeruginosa* karena adanya gangguan oleh *pyocins* yang terdapat intrinsik pada spesies ini.

Typing dengan spektroskopi Raman menghasilkan lima klaster utama CNAB, klaster terbesar (CIPTO-31) terdiri dari isolat yang berasal dari pasien (skrining dan spesimen klinis) dan isolat dari lingkungan. MLST yang dilakukan untuk 14 isolat, menghasilkan adanya beberapa tipe sekeuess (*sequence types* (STs)), yang sesuai dengan pengelompokan spektroskopi Raman. Empat

jenis ST yang sebelumnya sudah diidentifikasi (ST195, ST208, ST218, dan ST642) serta beberapa ST baru, dan alel baru untuk *gen gpi* ditemukan dalam penelitian ini. (2)

Analisis spektroskopi Raman juga menghasilkan adanya beberapa klaster CNKP. Ada tiga klaster utama, klaster terbesar (CIPTOKPN24) terdiri dari isolat yang diperoleh dari pasien (skrining dan spesimen klinis) dan ditemukan di kedua ICU sepanjang masa studi, sedangkan klon lain tampaknya bervariasi pada waktu tertentu. (4) Sebanyak 97 isolasi klinis dan 1 isolat dari lingkungan dianalisis lebih lanjut menggunakan metode *genoyping* MLVA, teridentifikasi 30 genotipe yang berbeda. Pengelompokan strain oleh spektroskopi Raman menjadi tiga kelompok dominan sesuai dengan pengelompokan oleh MLVA, misalnya 20 strain Raman CIPTOKPN24 semuanya merupakan milik satu kompleks klonal MLVA. Demikian juga keempat strain Raman CIPTOKPN30 milik satu kompleks klonal MLVA seperti yang ditemukan pada isolat CIPTOKPN27. (4)

MLST mengungkapkan empat klaster utama CNPA (ST235, ST823, ST446 dan ST357) serta beberapa *sequence types* baru. Pada MLVA, lima klaster utama dibedakan menjadi dua milik ST235 dan yang lainnya sesuai dengan ST823, ST446 dan ST357. Sebagian besar isolat milik ST235 (isolat pasien dan lingkungan), di mana 22 isolat memiliki gen bla_{IMP}, 24 isolat memiliki gen $bla_{\text{GES-5}}$ tetapi tidak ada isolat yang mengandung gen bla_{VIM}. Semua ST823 memilki gen bla_{VIM}.

Kami selanjutnya mengevaluasi lima jenis teknik *typing* molekuler dengan menggunakan isolat CNPA yang secara epidemiologis yang sudah diketahui karakteristiknya. Teknik tersebut adalah sebagai berikut *Multi-Locus Variable Number of Tandem Repeat* (VNTR) *Analysis* (MLVA*), in-silico seven-loci multi-locus sequence typing* (MLST), *core and whole genome MLST* (cg/wgMLST), *core Single Nucleotide Polymorphism* (SNP) (cgSNP). Temuan kami menunjukkan bahwa tiga teknik terakhir (cgMLST, wgMLST dan cgSNP) memberikan tingkat resolusi tertinggi yang memungkinkan analisis epidemiologi lebih rinci dari wabah lokal dan penyebaran internasional. MLVA adalah alternatif yang cocok untuk *typing P. aeruginosa* yang akurat, berguna dalam kondisi transisi bila WGS tidak bisa dilakukan.

Dengan demikian, kesan secara keseluruhan adalah bahwa strain *carbapenem-non susceptible* dari tiga spesies yang ditargetkan oleh penelitian kami menunjukkan adanya endemisitas beberapa klon yang beredar di antara pasien ICU dan lingkungan ICU.

Tujuan 2 dari penelitian ini: *Untuk mengembangkan metode intervensi yang dapat untuk diterapkan dalam seting ICU sumber daya terbatas - yang dapat secara signifikan mengurangi risiko akuisisi dan infeksi oleh carbapenem-non-susceptible A. baumannii, P. aeruginosa* dan *K. pneumoniae.*

10

Sebagian besar HAI dapat dicegah melalui langkah-langkah pencegahan dan pengendalian infeksi (IPC) yang efektif. (11) Setelah fase 1, kami memperkenalkan bundel multimodal intervensi IPC yang awalnya terdiri dari langkah-langkah berikut (12) dan melihat bab 8:

1. Program multifaset peningkatan kepatuhan kebersihan tangan.
   Berdasarkan program kebersihan tangan yang terdapat dalam pedoman *Five Moments for Hand Hygiene* WHO. Program peningkatan kepatuhan kebersihan tangan mencakup edukasi dengan mengujia pengetahuan dan sikap pra-dan pasca-kuesioner, umpan balik kinerja dan pengingat, wawancara, dan *role-models* seperti yang telah dijelaskan sebelumnya. (12)
2. Satu putaran kampanye desinfeksi lingkungan yang melibatkan seluruh lingkungan di kedua ICU dengan menggunakan larutan natrium hipoklorit 1:100. Larutan disinfektan ini digunakan pada dinding, lantai, pintu, tempat tidur (kasur dan rel tempat tidur), wastafel, meja disamping, pompa infus dan pompa hisap dan tiang, monitor dan ventilator termasuk kabel penghubung, di bagian atas meja termasuk ruang cuci yang berdekatan. Selain itu, semua tirai di antara tempat tidur ditukar dengan yang bersih.
3. Desinfeksi lingkungan rutin dengan larutan natrium hipoklorit 1:100 yang mencakup lantai, tempat tidur, dan lingkungan sekitar pasien. Ini dilakukan dua kali sehari. Untuk kotoran yang terlihat, pertama kali dibersihkan dengan sikat dan air, sebelum menggunakan larutan natrium hipoklorit. Prosedur intensif seperti yang dijelaskan di atas diulang setiap dua minggu dalam fase ini. Tirai di antara tempat tidur diganti setiap 1-2 bulan atau segera bila ada kotoran terlihat.
4. Pemberlakuan penatagunaan antibiotik (termasuk evaluasi harian semua resep antibiotik pada hari kerja).
5. Semua pasien yang ditemukan positif untuk satu atau lebih *carbapenem-non- susceptible A. baumannii-calcoaceticus* complex, *K. pneumoniae*, atau *P. aeruginosa* dikohort di salah satu sudut khusus di ICU. HCW mengenakan masker, gaun, dan sarung tangan ketika mendekati dan memberikan perawatan untuk pasien yang kohort.
6. Mandi / membersihkan seluruh tubuh setiap hari dengan kain/washlap yang sudah mengandung larutan chlorhexidine gluconate 2%. Washlap ini disiapkan dan dikemas secara individual dalam kemasan plastik tertutup oleh unit farmasi rumah sakit.
7. Memperkenalkan larutan glukonat klorheksidin 2% untuk dekontaminasi orofaring. Botol yang mengandung larutan ini juga disiapkan dan disediakan oleh unit farmasi rumah sakit dan digunakan per pasien. Dekontaminasi oral dilakukan 4 kali setiap hari.

Tujuan 3 dari penelitian: *Untuk menerapkan dan menentukan efikasi intervensi (dikembangkan seperti yang telah dijelaskan di atas / di tujuan nomer 2) di seting ICU sumber daya terbatas.*

Kami mengukur pengetahuan *hand hygene* (HH) dan kepatuhan HH sebelum (di fase dasar) dan langsung setelah program multifaset (paska-intervensi) dan melakukan evaluasi ulang tiga tahun kemudian. Program peningkatan multifaset meliputi pendidikan, umpan balik, pengingat, wawancara dan penggunaan *role-models*. Ada peningkatan yang signifikan secara statistik dalam skor median pengetahuan HH secara keseluruhan pada paska-intervensi. Kepatuhan HH secara keseluruhan adalah 27% pada fase dasar dan secara signifikan meningkat menjadi 77% paska-intervensi. Untuk kelima momen HH, kepatuhan perawat dan dokter secara terpisah meningkat secara signifikan dari fase dasar ke fase paska intervensi, kecuali untuk "momen 3" (setelah paparan cairan tubuh), di mana pada fase dasar sudah tinggi. Sebagian besar tingkat kepatuhan secara signifikan lebih rendah di kedua kelompok petugas kesehatan setelah evaluasi tiga tahun kemudian, mereka pada dasarnya kembali ke tingkat pra-intervensi. Secara keseluruhan, kepatuhan HH terhadap perawat secara signifikan lebih baik daripada kepatuhan dokter. (12) Dengan demikian, menjaga kepatuhan HH tinggi memerlukan pemantauan yang berkelanjutan dan intervensi rutin.

Kami mengevaluasi efek intervensi terhadap akuisisi *carbapenem-non-susceptible Acinetobacter baumannii-calcoaceticus* complex (CNAB), *Klebsiella pneumoniae* (CNKP), dan *Pseudomonas aeruginosa* (CNPA). Menggunakan studi desain kuasi-eksperimental sebelum dan sesudah, secara keseluruhan untuk ketiga spesies ada "step change" yang signifikan, dari fase 1 ke fase 3 dalam tingkat akuisisi strain *carbapenem-non-susceptible.* Penurunan signifikan dalam tingkat akuisisi keseluruhan strain *carbapenem-non-susceptible* dari ketiga spesies terutama disebabkan oleh penurunan akuisisi *carbapenem-non-susceptible A. baumannii-calcoaceticus* complex dan, pada tingkat yang lebih rendah, *K. pneumoniae.* Menariknya, tingkat akuisisi *P. aeruginosa* hanya sedikit dipengaruhi oleh intervensi multimodal. Dalam masing-masing dari dua fase tidak ada tren ke penurunan atau peningkatan utama yang diamati dalam tingkat akuisisi strain resisten untuk salah satu dari tiga spesies secara terpisah atau untuk tiga spesies yang diambil bersama-sama, meskipun risiko akuisisi meningkat sedikit di fase 1. (lihat bab 8)

Penggunaan WGS yang dikombinasi dengan data klinis, kami dapat melacak dengan akurat penyebaran endemik *carbapenem-non-susceptible* strain *Pseudomonas aeruginosa* selama periode 3 tahun di ICU. Kami menemukan bahwa jumlah transmisi dan akuisisi CNPA oleh pasien sangat bervariasi dari waktu ke waktu, tetapi secara keseluruhan, lajunya memang tidak berkurang secara signifikan oleh intervensi. Sumber di lingkungan ICU terlibat dalam transmisi dan akuisisi ini. Empat klon CNPA internasional berisiko tinggi (ST235, ST823, ST357, dan ST446) mendominasi, tetapi distribusi klon ini berubah secara signifikan setelah intervensi dilaksanakan. (6) Dengan demikian, intervensi multimodal dapat mengubah komposisi klon *carbapenem-non-susceptible P. aeruginosa* tetapi tidak membasmi sumber ataupun mempengaruhi transmisi strain tersebut di lingkungan ICU.

Kami menyimpulkan bahwa intervensi multimodal yang bertujuan untuk mencegah akuisisi strain resisten patogen ICU merupakan usaha yang penting, dan pada dasarnya mampu dan mungkin cukup efektif untuk ICU di negara-negara berpenghasilan menengah ke bawah. Namun, intervensi multimodal berkemungkinan tidak sama efektifnya untuk semua spesies yang strain antibiotik-resisten.

REKOMENDASI UNTUK PENELITIAN SELANJUTNYA

Studi kami dilakukan di dua ICU hanya di satu rumah sakit rujukan nasional di Jakarta, Indonesia. Karena Indonesia adalah negara yang luas dan terpadat keempat di dunia, data yang disajikan dalam tesis ini tidak dapat mewakili seluruh Indonesia.

Di masa depan, program surveilans nasional untuk bakteri batang Gram-negatif negatif *carbapenem-non-susceptible* harus ditetapkan, di seluruh provinsi di Indonesia, dengan pengumpulan data epidemiologi, mekanisme resistensi fenotipik dan genotipik, dan analisis terkait kloning. Sistem deteksi awal harus diatur untuk pengenalan klon "berisiko tinggi" atau rute transmisi yang tanpa tersembunyi. Misalnya, adanya temuan kami bahwa beberapa pasien sudah membawa isolat *carbapenem-non-susceptible* pada saat masuk ke ICU, bisa menjadi potensi untuk penularan antar rumah sakit ketika pasien dirujuk, atau bahkan kemungkinan adanya keberadaan reservoir di masyarakat atau komunitas. Semua potensi Sumber dan rute penularan ini harus dieksplorasi atau diteliti lebih lanjut.

Kami menemukan variasi yang terbatas pada gen karbapenemase di setiap spesies, CNAB hanya memiliki *gen* $bla_{\text{oxa-23}}$ dan $bla_{\text{oxa-51,}}$ CNKP hanya membawa bla_{NDM} dan CNPA membawa bla_{VIM}, $bla_{\text{GES-5}}$ dan $bla_{\text{IMP..}}$ Apakah kita juga dapat menemukan gen pengkodean karbapenemase yang sama di antara isolate ICU dari ketiga spesies ini di provinsi lain di Indonesia? Itu masih dipertanyakan. Sistem surveilans akan menghasilkan data tentang hal ini.

Kami menyimpulkan bahwa intervensi multimodal yang bertujuan untuk mencegah akuisisi strain resisten patogen ICU yang penting, mampu dan mungkin cukup efektif dalam ICU di negara-negara berpenghasilan menengah ke bawah, tetapi tidak untuk CNPA. Pembersihan lingkungan tampaknya menjadi bagian yang sangat penting dari intervensi dengan CNPA. *Pseudomonas aeruginosa* adalah patogen yang unik, organisme ini dapat bertahan hidup selama periode jangka panjang di lingkungan yang lembab. Ada dua kemungkinan jalur transmisi untuk CNPA, yaitu melalui paparan air (limbah) yang tidak diproses dan melalui wastafel / keran yang terkontaminasi di rumah sakit. Hipotesis ini perlu dieksplorasi oleh upaya penelitian kolaboratif multicentre.

Kami menemukan dalam penelitian ini, bahwa penggunaan antibiotik, terutama karbapenem, di ICU adalah faktor risiko independen utama untuk akuisisi strain *carbapenem-non-susceptible*. Oleh karena itu, kami menyarankan bahwa penatagunaan antimikroba harus

diperkenalkan dalam praktik sehari-hari di ICU danefeknya pada epidemiologi resistensi antimikroba dipelajari pada seting ini.

Juga, penerapan intervensi tidak dipantau kecuali untuk kepatuhan kebersihan tangan. Studi di masa depan harus memonitor pembersihan lingkungan dengan cermat (dengan pengamatan praktik pembersihan, dan dengan lebih banyak kultur lingkungan), memantau penggunaan klorheksidin untuk dekontaminasi tubuh dan oral) dan yang terakhir, harus dipantau apakah pasien dengan kultur positif benar-benar dilakukan kohort di salah satu sudut ruangan atau ruangan yang terpisah. Ini semua harus dicatat dalam studi di masa depan, pada dasarnya memberikan data yang akurat tentang pelaksanaan intervensi multimodal.

Akhirnya, ketika membangun fasilitas ICU baru, bahkan dalam seting sumber daya terbatas, seseorang harus lebih memperhatikan untuk menghindari terciptanya lingkungan khas untuk patogen ICU dan memberikan lebih banyak hambatan struktural untuk kelangsungan hidup dan penyebaran mikro-organisme pada umumnya.

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APPENDIX

A

Acknowledgements
Curriculum Vitae
PhD Portofolio
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Publications

ACKNOWLEDGMENTS
UCAPAN TERIMA KASIH

First of all, I would like to express my gratitude to Allah SWT, who has given the opportunity on the amazing PhD journey and very memorable chapter in my life. It was a journey with many bloody tears and sweats, sadness, and happiness episodes. Trying to survive at the worst time in my life, lost both of my parents, almost gave up**. But Finally... I FINISHED IT!! Alhamdulillah!!**

First, I would like to thank the Republic of Indonesia government, especially the Directorate General of Higher Education of the Ministry of Education and Culture of the Republic of Indonesia, for granting a doctoral DIKTI-NESSO scholarship grant to conduct my PhD study in Rotterdam.

I am grateful to the present and former Rectors of Universitas Indonesia, for giving me permission and support to conduct my Ph.D. study.

I also would like to express my appreciation to the current and former Deans of the Faculty of Medicine Universitas Indonesia, Prof. Dr. dr. Ari Fahrial Syam, SpPD-KGEH, MMB, and Prof. Dr. dr. Ratna Sitompul, SpM(K) for granting permission and provided support for my Ph.D. study. Furthermore, I also would like to express my sincere gratitude to the Directors of Cipto Mangunkusumo National Referral Hospital in Jakarta, Indonesia, where I performed the studies in my thesis.

Prof. Henri. A Verbugh, my promotor, I don't know how to acknowledge and express my gratitude to you. You and Mrs. Annemick were like my parents, my best friends, but at the same time, also my big boss. We can laugh together, discuss and debate on many things, did many fun activities together, ate many martabak, and enjoyed many coffee time. You taught me how to deal with my life, even at the worst time. You shared your knowledge without any limitations. Furthermore, you are always patience when I made mistakes and in a "lemot" mode. I remember the first time we met in 2008. The big challenge in my life was travelling to Rotterdam alone for the first time. You introduced me with a "strippen" card for my favorite Tram 8, how to enjoy my life as a Rotterdammers, and how to forget "macet" (traffic jam) and "jam karet". I am also deeply grateful to all your family members, who always treated me with kindness, especially when I come to your house in the weekends during my time in the Netherlands. Thank you big boss. Hopefully, we can further continue our wonderful collaboration in the future till death do us part. This paragraph is not enough to express my gratitude to you.

I also would like to expres my gratitude to my co-promotor , Dr. Juliette. A. Severin, Ph.D. Honestly, when we met for the first time, I was a little bit afraid. You are a very serious person, smart, and look to details. It's all gone after getting to know you more. You guide me patiently and discuss our project intensely and how to adapt to the laboratory work flow. Your passion and enthusiasm as a clinical microbiologist always inspire me, and you have stimulated me to be a better researcher and an ideal clinical microbiologist. Thank you to your family too (Marcello and the kids) for all of the hospitality during my stay in the Netherlands; I hope our fruitful collaboration will continue in the next project.

Dr. Anis Karuniawati, Ph.D., SpMK(K), you are not only my co-promotor, but also a big sister that I never had. Your support means a lot to me and you always stand beside me. Momen yang tidak pernah terlupakan ketika saya mengabari akan operasi… thanks dok… saya bisa tegar dan kuat sampai pada pencapaian sekarang ini.

I would like to thank Prof.dr. Annelies Verbon, Prof.dr. D. de Jong, Prof.dr. A. Voss, Prof.dr. A. van Belkum, Prof.dr. Kuntaman, Prof.dr. M.J.M. Bonten, Prof.dr. H.F.L. Wertheim and, Prof.dr. H.P. Endtz as the the members of the doctoral committee of my thesis.

Prof. dr. Pratiwi Sudarmono, Ph.D, SpMK(K), thank you for your support. You are a good role model and one of my motivations to be a good clinical microbiologist. Tolong jangan pensiun dulu.. saya masih butuh nasihat dari Prof.

I am also profoundly grateful to all the co-authors and collaborators, especially to Prof. Alex Van Belkum, Ph.D., Ph.D.; thank you for being such a supportive work-group leader. Hopefully, we can continue our collaboration with some projects already on plan. I also would like to thank Andreu Coello Pelegrin, Caroline Mirande, and H.Qi for the collaboration projects, resulting in this thesis's papers.

Prof. Dr. dr. Kuntaman, SpMK(K), as a Head of PAMKI committee, Prof. dr. Usman Chatib Warsa, Ph.D., SpMK(K), Prof. dr. Amin Soebandrio, Ph.D., SpMK(K), Prof. dr. Agus Sjarurahman, Ph.D., SpMK(K), I am grateful that I could be a staff of the Department of Clinical Microbiology FMUI and obtained a lot of knowledge from you, starting from my time as a clinical microbiologist fellow until now.

To all of PAMKI Indonesia and PAMKI Jakarta members, thank you for all of your support especially to dr. Dewi Anggraini, SpMK(K), Dr. dr. Wani Gunardi, SpMK(K), and dr. Dewi Santosaningsih, SpMK

Dr. Fera Ibrahim, MSc, Ph.D., SpMK(K), Head of the Department Microbiology Faculty of Medicine Universitas Indonesia/ Dr. CiptoMangunkusumo Hospital, thank you for your continuous support and encouragement. But moreover, thank you for your patience when I am in a bad mood.

Dr. Rudyanto Sedono, SpAn-KIC, Dr. dr. Dita Aditianingsih, SpAn-KIC, dan semua sejawat dan teman-teman di ICU dewasa; Mpok Dedeh, Mak Uwo Yumna, Kak Sherina, Tante Sondang, Mela dan ICU IGD; Bu Nur,Mba Ooh, Mba Ely terima kasih sudah mengizinkan saya "mengubek-ubek" isi ICU dan membantu dengan sepenuh hati semua penelitian dan selama masa studi saya.

I also would like to thank all of my colleagues at the Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam. Prof Annelies Verbon, as the Head of the Department, I am grateful for your help and support throughout the years of my Ph.D. study. I hope the collaboration between Rotterdam and Jakarta will continue in the future.
Prof Hubert Endtz, I would like to thank you for your willingness to be a doctorate committee member and an effort to review my thesis. Dr. Jan Nouwen, you've been accommodating throughout my time at Erasmus MC, and I am deeply thankful for that. I hope we can continue our collaboration in the future, either through Minor Global Health or other projects between FKUI and Erasmus MC. Dr. Will Goessen, thank you for your help with my study project and my thesis. Dr. Corné HW Klaassen, Ph.D., thank you for your collaboration, introduced me to MLVA and advanced molecular technique. I hope we can collaborate again in the future and I will bring more "Rendang" for you. Dr. Willem J.B van Wamel, thank you for your support during my study period. To the secretaries, Simone Jongbloed and Marian Humphrey, I am grateful for your help and support throughout the years of my Ph.D. study. Without both of you, getting my Ph.D. would be much more difficult. Special thanks to Jannete, thank you for your willingness to be my paranymph, and all of fruithfull discussion and assistance during my PhD journey. Special thanks to Wilson, for eliminating the lonely feeling that sometimes occured.

Diagnostic Unit of MMIZ; A. Luitendijk, thank you for your help with all of the technical support during my Ph.D. study. Willemine, Lisbet, thank you so much for your help with the lab works, especially the MLVA project.

Unit Research and Development MMIZ; Nicole Lemmens, Dennise M.C. Vermeulen - de Jongh thank you for introducing me to molecular laboratory work, Deborah Horst-Kreft thank you for your help with my collection strain, Carla Roodbol-de Goeij...your passion about the logistic was impressed me, Mehri Tavakol, Susan Snijder, thank you so much for your help with the lab works. I am grateful to Diana Wilemse-Erix for analyzing and consultation regarding the Raman spectroscopy.

Michelle de Regt, Zelly Dubber, Ayse Demir, thank you for help with the laboratory work. Damiat Aoulad Fares, Souhaib El-Atmani, the medical student from Erasmus University Medical Centre Rotterdam, who helped collected data, analyze, and as a co-author on Hand Hygiene Multimodal Intervention project. Thank you very much for our collaboration. It was fun working with you guys.

Dr. Raoul Tan, thank you for your attention and help throughout my PhD study. We often discuss how to improve the collaboration between FKUI and Cipto Mangunkusumo Hospital with Erasmus MC. I hope that all your efforts will become fruitful in the near future and regards to Eno and the kids.

Thank you and my gratitude to all of staf Department of Clinical Microbiology Faculty of Medicine Universitas Indonesia; Terima kasih tak terhingga kepada seluruh staf Departemen Mikrobiology Klinik FKUI/RSCM; Dr. dr. Mardiastuti, MS, SpMK(K) .. always smile and be patient to me,,,, Dr. T. Mirawati Sudiro,Ph.D., Dr. dr. Budiman Bela, SpMK(K), Dr. dr.Yeva Rosana, MS, SpMK(K), Beti Ernawati,Ph.D., Andriansjah Roekmana, M.Biomed, Ph.D., Dr. Andi Yasmon, M.Biomed, Fithriyah, M.Biomed, Ph.D., Dr. Conny R. Tjampakasari, M.Biomed., Dra Ariyani Kirasari, M.Biomed., Dra. Ikaningsih, M.Biomed., Dr. Delly Chipta Lestari, SpMK(K), Dr. Ardiana Kusumaningrum,SpMK(K), Dr. Angky Budianti, SpMK(K), dan Dr. Dimas Seto, SpMK(K).

Kepada seluruh analis dan karyawan UKK Laboratorium Mikrobiologi Klinik FKUI, Mba Aisyah dan Bu Tati, terima kasih sudah sangat membantu untuk penelitian saya sejak pertama kali saya di LMK, Bu Elizabeth, pak Aan, Ika, Lindah, Ayub, Mariah, Karima, Bu Lina, Shinta, Mba Ice, Mariana, Toyo, Ai, Mba Esty, Mba Henny, Mba Yatmi, Novi, Mba Mala, Mba Tita, Iphel, Imah, Nida, Indri, Rela, Lola, Nurul, Adel, Savitri, Alfian, Helen, Hetty, Koko, Willy, Tomo, dan semua karyawan, dan keluarga besar UKK LMK FKUI yang tidak bisa saya sebutkan satu persatu.

To all the Clinical Microbiology Fellows of FMUI, thank you so much for your help guys. Best wishes with your study and future career.

Thank you to the Director and management team of Universitas Indonesia Hospital (RSUI), Infection Prevention Committee (Ns. Agustin and Ns. Bhekti).

Thank you to the Director and management team of Pelni Hospital Jakarta.

Terima kasih yang tidak terhingga dan mohon maaf yang sedalam-dalamnya kepada Guru besar, senior, teman-teman, dan saudara yang tidak dapat saya sebutkan satu persatu di dalam ucapan terima kasih ini.

APPENDIX

Special thanks to the special people that have been supportive and have been like my second family in the Netherlands: Bang Syam and Atika, Om Tinus and Tante Ade, Tante Meike and Om Andre, and all of family member.

Word cannot express how grateful I am for having good Indonesian friends during my time in the Netherlands. To my band of brothers and sisters in the Netherlands: Teh Dowty and Kang Oppik; my ibu and bapak kos, please forget about diet and gym; let's enjoy our life with many wonderful "things" in our fridges. Om Asjone, Walikota Rotterdam versi student, thank you for your hospitality dan ga bosan direpotin sama Uni yaa. Rina; my housemate and my sister, too many stories that we have and thank you for the good time during our stay in Rotterdam. The Mullerkade apartment is a historical witness to the ups and downs of our lives in Rotterdam. Dagdo; the genius but at the same time the weird and annoying person, always be yourself Do, no matter what people said. Adhit; the cutest and gorgeous person in Rotterdam (kata dia!!), still be a humble person, don't forget with your passion (Vogue editor..LOL), success with your career and romance. Raya (bang Haji), the richest person among us, still be a nice person, but sometimes "julidnya" ga ketulungan, thank you for your hospitality. Bri… the Mulan princess, thank you for being bothered with all kinds of matters. Anfasa Family (Fasa, Salma, Princess Safa, and Prince Fatih), ga tau bagaimana mengucapkan terima kasih kepada kalian, yang selalu mau direpotin, yang selalu mendengarkan omelan dan curhatan, yang selalu siap menolong bawain belanjaan (IKEA memory). I love you guys, and I hope we are always a family, forever and ever. Ahmad Fuady, thank you always for being the statistician consultant, regards to Diah and children. Rofyan, thank you for all of fruithfull discussion in MMIZ dan selalu menghibur dengan gossip teranyar nya dan selalu siap menolong ibu-ibu PhD yang kadang lemotnya ga ketulungan di lab… sukses dengan SpMK nya. Hakim, sukses juga untuk karir ke depannya, Mas Adi Nugroho, ..kapan nih kita jalan lagi bertiga,,,,ternyata terbukti kalian berdua ga serius-serius amat hahahaha….mbak Alfi, Ajeng, dan teman-teman seperjuangan beasiswa DIKTI NESO batch pertama dan terakhir… sukses guys…. Dicky dan Maria terima kasih sudah mau direpotkan sampai sekarang untuk konsultasinya, dr Karyanti, Henny, Irene, Putri terima kasih sudah selalu menjadi teman weekend, teman traveling, teman makan, teman curhat....terbaiklah…. having pleasure to know you guys….

To all of my big family, Saya ingin mengucapkan penghargaan dan terima kasih sebesar-besarnya kepada seluruh keluarga besar saya yang telah mendukung dan selalu mendoakan saya; Kelg besar M.Said (klg Awet & Atong, Klg Granny & angku Bas, Klg Opa Jang & Oma Meme, Klg Alm Om Aufa & One, Klg Om Yus & Tek Yu), Klg besar Leman Dt. Bandaro (om Rocky& budhe Elly, klg Prawoto Sunu, Mba Ning& Mas Sofyan),

Keluarga besar Ibrahim Nurdin (bapak mertua alm. Ibrahim Nurdin & ibu almh. Ramayulis, terima kasih telah menerima saya sebagai menantu yang jauh sekali dari ideal) . Terima kasih pula kepada kakak ipar saya,dan seluruh keluarga besar yang tidak bisa saya sebutkan satu persatu. Semoga Allah SWT memberikan balasan atas kebaikan dan perhatian yang diberikan.

Adek-adek ku Devi Armand Saharman, Bob Adrian Saharman dan Kathrin. Terima kasih sudah membantu menjaga papa&mama, dan anak-anak selama uni ga ada.

Sembah sujud saya untuk Almarhum kedua orang tua saya Alm. papa Saharman Leman dan Almh. mama Gusniar Said yang selalu mensupport saya, selalu berada di samping saya walau apapun yang terjadi. Papa adalah panutan saya, my role model. Kasih sayang papa dan mama sepanjang masa dan tidak terbalaskan. Pesan papa yang selalu menjadi motivasi saya "Kesempatan baik belum tentu datang dua kali". Al Fatihah to my parents who already passed away during my PhD journey. Semoga kita dapat berkumpul kembali di Surga Nya...

To my soul, my precious, my ticket to Jannah; Faishal Farras Yanfaunnas, Atika Mahira Yanfaunnas, Faris Atha Muhana Yanfaunnas.. Maafkan mama... bila raga ini tidak bisa selalu hadir menemani kalian dalam suka dan duka…Maafkan mama... bila tangan ini tidak bisa selalu memeluk kalian... Maafkan mama… bila bahu ini tidak bisa selalu menjadi sandaran kalian…Doa mama selalu dalam setiap helaan nafas dan sujud mama. Semoga kalian bertiga menjadi anak yang sholeh dan sholehah dan sukses di dunia dan akhirat. Aamiin Allahumma Aamiin

Last but not least, to my partner in crime for 25 years, my backbone, my shoulder to cry on… Yanfaunnas… terima kasih sudah mengizinkan Lili menggapai cita-cita, terima kasih sudah mau bertahan di sisi Lili dalam suka dan duka, terima kasih selalu mensupport dalam situasi apapun juga, terima kasih sudah merawat dan menjaga anak-anak kita sepeninggal Lili, terima kasih sudah selalu menjadi "main supporting system", terima kasih sudah menyayangi dan memilih Lili sebagai pendamping hidup, maafkan kalau Lili belum bisa menjadi seorang istri yang shalehah dan menjadi ibu teladan untuk anak-anak kita. I love you to the moon and back.

Finally, eventhough it's the end of my journey, I will never forget you .. **ROTTERDAM**, thank you with all of the hospitality and amazing experiences. I am really ... really… enjoyed my time with you: Tram 8, Akong, Anugerah, Round & Round, Bazaar, Poffertjes, Patat, Oriental, Het Park, Erasmus Bridge, Blaak, Markthaal, Lijnbaan. It's been wonderful also to be able to visit the "famous" Roermond and the other hidden gems in the Netherlands…. I will miss you so much and I promise that I will come back in the future.

Curriculum Vitae

Yulia Rosa Saharman was born in Padang, West Sumatera, Indonesia, on July 18, 1970. She was raised by her parents, Saharman Leman and Gusniar Said, and grew up together with his two younger brothers (Devi Armand Saharman and Bob Adrian Saharman).

In 1989, She studied medicine at the Faculty of Medicine, Universitas Andalas (FM Unand) Padang finishing it in 1996 with honors. Afterwards, he was working as a general practitioner at Air Tawar Primary Health Care Unit in Padang, West Sumatera (1996-1999). She was also working as a lecturer and researcher in the Departement of Microbiology, Faculty of Medicine, Universitas Andalas Padang, West Sumatera from 1998-2008. Subsequently, she continued doing residency at the Department of Microbiology FMUI – Cipto Mangunkusumo National Referral Hospital in 2004. She graduated as a clinical microbiologist specialist in 2008 with a cum laude award. Subsequently, she moved to Jakarta and was accepted as a lecturer and researcher in the Departement of Microbiology, Faculty of Medicine Universitas Indonesia.

She obtained a fellowship opportunity to do a training in consultative aspect of clinical microbiology at Erasmus Medical Centre, Rotterdam in 2009 under the supervision of Prof.dr. Henri A. Verbrugh. This led to an opportunity to do a PhD study, which he started in 2012, under the supervision of Prof.dr. Henri A. Verbrugh, Dr. Juliette A Severin, and Dr. Anis Karuniawati, Ph.D., SpMK(K), at the Department of Medical Microbiology and Infectious Disease, Erasmus MC. This study was funded with a DIKTI NESSO PhD Scholarship grant from The Directorate General of Higher Education of the Ministry of Education and Culture of the Republic of Indonesia and internal grants from the Department of Medical Microbiology and Infectious Disease, Erasmus MC. She has been appointed as the vice head of clinical microbiology laboratory of Faculty of Medicine Universitas Indonesia since 2018. Furthermore, she is also a member of the antimicrobial resistance control committee of Cipto Mangunkusumo National Referral Hospital. She has also been working at Rumah Sakit Universitas Indonesia (RS UI), which is the newly established teaching hospital of Universitas Indonesia, since 2018. Currently, she is the head of infection prevention committee of this hospital.

She is married to Yanfaunnas and is a proud mother of Faishal Farras Yanfaunnas, Atika Mahira Yanfaunnas, and Faris Atha Muhana Yanfaunnas.

PhD Portfolio

Name	Yulia Rosa Saharman
Department	Medical Microbiology and Infectious Disease
Research school	Postgraduate school molecular medicine (PGS Molmed)
Ph.D. period	2012-2020
Promotors	Prof. dr. Henri.A Verbrugh
Co-promotor	Dr. Juliëtte A. Severin
	Dr. Anis Karuniawati, Ph.D., SpMK(K)

EDUCATION BACKGROUND	
2012-now	PhD candidate in Medische Microbiologie & Infectieziekten Erasmus MC , Rotterdam, the Netherlands
2004- 2008	SpMK (Clinical Microbiologist Spesialist) Department of Microbiology, Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia
1994-1996 1989-1994	Medical Doctor (MD) Bachelor of Medicine (S.Ked) Faculty of Medicine, University of Andalas, Padang, West Sumatera
1986-1989 1983-1986	SMAN 1 Padang (High School) SMPN 2 Padang (Junior High School)

TRAINING, COURSE, SEMINAR	
Oct 20th 2008- 16th 2009	Training in Consultative Aspect of Clinical Microbiology, Erasmus Medical Centre Rotterdam
11 October 2013	WS The Second Indonesian Clinical Microbiology Course, Semarang,
18-19 April 2013	The Second Indonesian Pharmacist Update Symposium Surabaya
26-27 April 2014	Symposium MD/XDR/TDR the Microorgamism of Pattern in VAP, 15th Jakarta Antimicrobial Update . Jakarta
Jakarta, 29-30 November 2014	"Antimicrobial Policies and Practices for Patient Safety" (9th National Symposium & Workshop of Indonesia Antimicrobial Resistance Watch and Annual Scientific Meeting of Indonesian Society for Clinical Microbiologist)
Jakarta, 28-29 November 2014	"Workshop on AST Customizes Card for Indonesia" (9th National Symposium & Workshop of Indonesia Antimicrobial Resistance Watch and Annual Scientific Meeting of Indonesian Society for Clinical Microbiologist)
Washington DC, 2014	ICAAC 2014
Jakarta, 2015	Open Science Meeting, IMERI, Jakarta
Rotterdam, 2015	Workshop on Microsoft Excel 2010: Basic, Erasmus MC 2015
Rotterdam, 2015	Workshop on Microsoft Excel 2010: Advnced, ErasmusMC, 2015
Jakarta, 6-8 August 2015	WS Systematic Review/Meta-Analysis,

Medan, 29 October 2015	WS of Rationale Interpretation of Antibiotic and Susceptibility testing
Amsterdam, April 2016	ECCMID 2016 Amsterdam, The Netherlands
Rotterdam, 2016	Research Integrity Erasmus MC 2016
Rotterdam, 28-30 November 2016	The Basic Introduction Course on SPSS Erasmus MC 2016
13 September,4 October,25 October, 3 November 2016	Biomedical Writing Course for MSc and PhD Student, Erasmus MC 2016
Rotterdam, 12-13 December 2016	Survival Analysis Course Erasmus MC 2016
Rotterdam, 14 December 2017	Science Day
Padang, October 2017	Annual Scientific Meeting (ASM) PAMKI – Pertemuan Ilmiah Tahunan (PIT)
Surabaya, 2018	Workshop Antimicrobial Resistance controle Update and Reviewer Audit of Antibiotics
Madrid, Spain, 21 – 24 April 2018.	28th ECCMID, the European Congress of Clinical Microbiology and Infectious Diseases,
Surabaya, 13-14 October 2018	Update on Management Prevention and Control of Infectious Diseases in the Era of Antimicrobial Resistance (PAMKI annual Meeting)
Denpasar, 6-7 March 2019	INDOHUN Prospective Master Trainer on Travel Medicine at Travel Medicine Training
Surabaya, 26-29 March 2019	WHO GLASS Surveillance Training
Malang, 12-15 September 2019	PAMKI Annual Meeting 2019
LABORATORY AND FIELD SUPERVISE OF STUDENT	
2014 (6 month)	Supervised 2 internship HBO student (laboratory work) 1. Michelle de Reght 2. Zelly Duber
2014	Supervised 2 undergraduate student Erasmus MC (collected data, intervention Hand Hygiene and as a co-author on HH manuscript) 1. Damiat Aoulad Fares 2. Souhaib El-Atmani
2016(6 month)	Supervised 1 internship HBO student (laboratory work) 1. Ayse Demir
2014-2020	Teaching and tutor undergraduate medical student FMUI
2014-2020	Tutor of clinical microbiology fellowship
2014-2020	Tutor of Clinical laboratory course on Bacteriology section

PRESENTATIONS

- Resistensi Antibiotik Ditinjau dari Mikrobiologi Klinik:
- **Yulia Rosa Saharman**
- Oral Presentation
- Pertemuan Ilmiah Berkala XII Ilmu Penyakit Dalam, Padang 11-12 Februari 2012 https://sipeg.ui.ac.id/ng/arsipsk/20190508-Cat-ce9cb5872440073df5b4bb84d7d2edad.pdf

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- Antimicrobial susceptibility pattern among non-fermenting gram negative bacteria in Jakarta, Indonesia
- Delly Chipta Lestari, **Yulia Rosa Saharman**
- Poster presentation
- Fourth International Conference on Infectious Disease Dynamics 19-22 November 2013, Amsterdam, The Netherlands

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- Molecular Characterization of *Acinetobacter baumannii* in a Large Intensive Care Unit in Jakarta, Indonesia: Endemic Situation of OXA-23-positive Clones:
- **Yulia Rosa Saharman**
- Poster session , ICAAC 2014 , Washington DC

- Kepatuhan Cuci Tangan di Unit Perawatan Intensif di RSUPN Dr. Cipto Mangunkusumo di Jakarta
- **Yulia Rosa Saharman**
- Oral presentation, Open Science Meeting Jakarta, 3 November 2015

- Endemic NDM-1-producing *Klebsiella pneumoniae* in a large intensive care unit in Jakarta, Indonesia:
- **Yulia Rosa Saharman**
- ePoster, ECCMID 2016 Amsterdam, The Netherlands.

- Carbapenem-nonsusceptible *Pseudomonas aeruginosa* in a Large Intensive Care Unit in Jakarta, Indonesia
- **Yulia Rosa Saharman**
- Oral presentation 14 December 2017, Science Day of MMIZ,

- Carbapenem-nonsusceptible *Pseudomonas aeruginosa* in intensive care unit in Jakarta, Indonesia.
- **Yulia Rosa Saharman**, A. Coello Pelegrin, A. Karuniawati, R. Sedono, D. Aditianingsih, W. Goessens, C. Klaassen, A. Van Belkum, C. Mirande, H. Verbrugh, J. Severin
- Oral flash session, 28th ECCMID, the European Congress of Clinical Microbiology and Infectious Diseases, Madrid, Spain, 21 – 24 April 2018.

- Transmission dynamics of carbapenem-nonsusceptible *Pseudomonas aeruginosa* in two ICUs of the national referral hospital in Jakarta (Indonesia): assessing the effectivity of an infection control intervention
- Andreu Coello Pelegrin , **Yulia Rosa Saharman** , Mattia Palmieri , Aurelien Griffon , Caroline Mirande , Wil Goessens , Corné H. Klaassen , Alex Van Belkum , Henri Verbrugh , Juliëtte A. Severin
- Poster session
- Event: ECCMID 2019 16 April 2019
- Amsterdam, The Netherlands

PUBLICATIONS

- Phenotype Characterization of Beta-laktamase Producing Enterobacteriaceae in the Intensive Care Unit (ICU) at Cipto Mangunkusumo Hospital in 2011
- **Yulia Rosa Saharman**, Delly Chipta Lestari
- ACTA MEDICA INDONESIANA - The Indonesian Journal of Internal Medicine Volume 45 Number 1 / January – March 2013

- Detection of carbapenemase encoding genes in Enterobacteriace, *Pseudomonas aeruginosa,* and *Acinetobacter baumanii* isolated from patients at Intensive Care Unit Cipto Mangunkusumo Hospital in 2011:
- Anis Karuniawati, **Yulia Rosa Saharman**, Delly Chipta Lestari
- Acta Med Indones. 2013 Apr;45(2):101-6.

- Efficacy of Intravitreal Levofloxacin 0,5% ophthalmic solution in treating *Pseudomonas aeruginosa* endophthalmitis in rabbit model
- **Yulia Rosa Saharman**, et al
- Journal of The Indonesian Ophthalmologists Association Vol. 40 No. 1 Jan-Juni 2013

- Endemic carbapenem-*nonsusceptible Acinetobacter baumannii-calcoaceticus complex* in intensive care units of the national referral hospital in Jakarta, Indonesia
  Yulia Rosa Saharman, Anis Karuniawati, Rudyanto Sedono, Dita Aditianingsih, Pratiwi Sudarmono, Wil H. F. Goessens, Corné H. W. Klaassen, Henri A. Verbrugh, Juliëtte A. Severin
- Antimicrobial Resistance and Infection Control (2018) 7:5

- A multifaceted hand hygiene improvement program on the intensive care units of the National Referral Hospital of Indonesia in Jakarta
- **Yulia Rosa Saharman**, Damiat Aoulad Fares,Souhaib El-Atmani, Rudyanto Sedono, Dita Aditianingsih, Anis Karuniawati, Joost van Rosmalen, Henri A. Verbrugh and Juliette A. Severin
- Antimicrobial Resistance and Infection Control (2019) 8:93

- Epidemiology and characterisation of carbapenem-non-susceptible *Pseudomonas aeruginosa* in a large intensive care unit in Jakarta, Indonesia
- **Yulia Rosa Saharman**, Andreu Coello Pelegrin, Anis Karuniawati , Rudyanto Sedono, Dita Aditianingsih, Wil H.F. Goessens , Corné H.W. Klaassen, Alex van Belkum , Caroline Mirande, Henri A. Verbrugh, Juliëtte A. Severin
- International Journal of Antimicrobial Agents 54 (2019) 655–660
- https://doi.org/10.1016/j.ijantimicag.2019.08.003

- High-Risk International Clones of Carbapenem-Nonsusceptible *Pseudomonas aeruginosa* Endemic to Indonesian Intensive Care Units: Impact of a Multifaceted Infection Control Intervention Analyzed at the Genomic Level
- Andreu Coello Pelegrin, **Yulia Rosa Saharman**, Aurélien Griffon, Mattia Palmieri, Caroline Mirande, Anis Karuniawati,Rudyanto Sedono,Dita Aditianingsih, Wil H. F. Goessens, Alex van Belkum, Henri A. Verbrugh, Corné H. W. Klaassen, Juliëtte A. Severin
- mBio . November/December 2019 Volume 10 Issue 6 e02384-19. https://doi.org/10.1128/mBio.02384-19.
- https://mbio.asm.org/

- Clinical impact of endemic NDM-producing *Klebsiella pneumoniae* in intensive care units of the national referral hospital in Jakarta, Indonesia:
- **Yulia Rosa Saharman**;Anis Karuniawati, Rudyanto Sedono, Dita Aditianingsih, Wil H. F. Goessens, Corné H. W. Klaassen, Henri A. Verbrugh and Juliëtte A. Severin
- Antimicrobial Resistance and Infection Control (2020) 9:61
- https://doi.org/10.1186/s13756-020-00716-7

- Preliminary report of COVID-19 testing: experience of the clinical microbiology laboratory Universitas Indonesia, Jakarta, Indonesia,
- F. Ibrahim, A. Natasha, **Y. R. Saharman** and P. Sudarmono
- New Microbes and New Infections Vol 37 Sept 2020 (Q3), ISSN : 20522975
- https://reader.elsevier.com/reader/sd/pii/S2052297520300858