*Prospective Clinical Research Report*

*Journal of* INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
2022, Vol. 50(9) 1–12

DOI: 10.1177/03000605221123678
journals.sagepub.com/home/imr
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# Identification of *Mycoplasma pneumoniae*-associated pneumonia cases among hospitalized patients using CLART® microarray technology

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## Abstract

**Objectives:** Community-acquired pneumonia (CAP) is a global health condition that affects populations from all age groups. The laboratory identification of *Mycoplasma pneumoniae* as a causative agent of CAP is challenging because of its atypical and fastidious nature. Therefore, this study assessed the diagnostic potential of PneumoCLART bacteria® in identifying *M. pneumoniae* as a causative agent of pneumonia in hospitalized adults.

**Methods:** This prospective study used a cross-sectional approach to assess the diagnostic potential of PneumoCLART bacteria® for detecting *M. pneumoniae* in sputum samples procured from 27 patients with pneumonia who required hospitalization.

**Results:** The PneumoCLART bacteria® results illustrated that 7 of 27 patients with pneumonia were positive for *M. pneumoniae* (26%). However, the quality of sputum varied among the *M. pneumoniae*-positive and *M. pneumoniae*-negative samples. Fifty percent of the specimens obtained from patients positive for *M. pneumoniae* were saliva-contaminated and unsuitable for analysis.

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**Conclusions:** Because the leukocyte count was low and sputum specimens were saliva-contaminated, these findings require further validation to prove the utility of CLART® microarray technology for the identification of *M. pneumoniae* in pneumonia-positive patients. Conclusively, this prospective study included a small number of clinical samples, which likely affected its outcomes.

**Keywords**
Community-acquired pneumonia, *Mycoplasma pneumoniae*, CLART® microarray technology, PneumoCLART bacteria®, sputum, hospitalization, saliva contamination

Date received: 5 December 2021; accepted: 15 August 2022

## Introduction

Community-acquired pneumonia (CAP) is a global concern that has crippled the healthcare systems of both developed and developing economies.[1–8] Reportedly, a variety of respiratory viruses and bacteria are associated with CAP, including human metapneumovirus, respiratory syncytial virus, influenza A, adenovirus, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, *Haemophilus influenza*, and *Staphylococcus aureus*.[4,9] Among these microbes, *M. pneumoniae* accounts for a fairly high percentage of CAP cases in adult and pediatric populations, and nearly half of the affected individuals require hospitalization.[4,10–14] Although *M. pneumoniae* is an important etiological agent of CAP, there are challenges associated with its diagnosis mainly because of its fastidious nature, seroprevalence, and possible transient asymptomatic carriage. Therefore, it is important to explore and develop efficient techniques for the detection of *M. pneumoniae* as an atypical pathogen of CAP.[15–17]

Because of their high sensitivity and specificity, multiplex molecular diagnostic modalities such as PneumoCLART bacteria® (Genomica, Madrid, Spain) allow pathogen-specific treatment and predict pathophysiological complications, making them methods of choice for detecting various respiratory tract pathogens. PneumoCLART bacteria® is particularly useful for detecting *M. pneumoniae*, a fastidious, slow-growing bacterial pathogen of the respiratory tract (incubation period is 7–21 days) with special growth requirements.[18–22] Noteworthy, PneumoCLART bacteria® permits the simultaneous detection and genotyping of multiple diagnostically challenging yet important respiratory tract bacterial pathogens, including *M. pneumoniae*, from uncultured clinical respiratory specimens (sputum, nasopharyngeal exudates/lavages/aspirates, bronchoalveolar lavage, and bronchial suction) in a single test. This in turn considerably reduces the turnaround time (up to 6 hours) and cost of the assay. Taken together, rapid bacterial detection enables the clinician to modify the antimicrobial therapy for *M. pneumoniae*, which is a crucial factor in improving patients' health and recovery prospects. Consequently, prolonged hospitalization and the use of ineffective regimens can be avoided, thereby reducing treatment costs. Another important aspect worth mentioning is that antibiotic therapy in patients does not affect their test results for the presence of

*M. pneumoniae* using PneumoCLART bacteria®, which is a nucleic acid-based PCR technique.[23] Although cell culture remains the gold standard for the laboratory confirmation of *M. pneumoniae*, its clinical utility is limited for the aforementioned reasons. In addition, culture techniques often fail to identify *M. pneumoniae*, which shares similar symptomologies with other crucial bacterial pathogens of atypical pneumonia, and it is mostly a co-infecting pathogen in CAP.[18–20,22] As a potential diagnostic and epidemiological tool, it is therefore pertinent to establish the clinical utility of PneumoCLART bacteria® by undertaking prospective studies for *M. pneumoniae* detection. Because the prevalence rate of *M. pneumoniae* as an agent of CAP in Indonesia is yet to be ascertained, this study assessed the positivity rate of *M. pneumoniae* from clinical specimens among patients with pneumonia using the PneumoCLART bacteria® method.

## Materials and methods

### Study outline and specimens

This cross-sectional, prospective study and collaborative work was conducted at Atma Jaya Hospital (Jakarta, Indonesia). The sputum samples of patients requiring hospitalization for pneumonia were included in this study. Consecutive sampling was performed for patients admitted to the aforementioned hospital between February 2017 and July 2017. Patients aged 18 years and older diagnosed with pneumonia by an attending doctor who could expectorate sputum were included in the study. Patients who declined to participate, failed to provide an informed consent form, or contracted other known causes of pneumonia such as active tuberculosis were excluded from this study. The routine bacterial cultivation and molecular methods using PneumoCLART bacteria® were performed simultaneously. The reporting of this study conforms to STROBE guidelines.[24]

### Sample size calculation

The sample size was calculated using the formula $n = \frac{Z\alpha^2 \times P \times Q}{d^2}$.

$Z\alpha$ is the conversion of the area under the normal curve at a certain confidence level against the standard deviation of 1.96 (when the accuracy interval applied was equal to 95%), $P$ is the prevalence rate of bacterial pneumonia in adults in Indonesia, which equals 4.5% (as revealed from the number of adults with pneumonia admitted to Atma Jaya Hospital), $Q$ equals $1 - P$, (0.955) and $d$ is the degree of desired precision (±10%), 0.1.

Using the aforementioned formula and values,

$$n = \frac{1.96^2 \times 0.045 \times 0.955}{0.1^2} = 16.5 \approx 17.$$

Twenty-seven research subjects were included in this study, slightly exceeding the calculated amount to account for potential sample mishandling.

### Research ethics and patient consent

The clinical specimens were obtained with the prior verbal informed consent of the patients.[25] This study was performed with the approval of the Departmental Ethical Committee of the School of Medicine and Health Science, Atma Jaya Catholic University of Indonesia (Ethical approval number: 11/05/KEP-FKUAJ/2017; approval date: 5 November 2017).

### Microbiology work-up, bacterial cultivation, and isolation

The routine bacterial cultivation was performed in fully equipped microbiology laboratories in the Microbiology Department,

Faculty of Medicine, Atma Jaya Catholic University (North Jakarta, Indonesia) following standard guidelines.[26] It is worth mentioning that bacterial cultivation was performed to identify pathogens other than *M. pneumoniae* (atypical pathogen) in the sputum samples and assess specimen quality opposed to being used as a comparative detection technique.

Post-culture, Gram staining of sputum samples was performed routinely before isolation to identify bacterial pathogens other than *M. pneumoniae* (which lacks a defined cell wall). Leukocytes and squamous epithelial cells (SECs) were identified and counted. The same bacterial growth media, namely chocolate agar, blood agar, and MacConkey agar (all from Sigma-Aldrich, Inc., St. Louis, MO, USA), were used in both hospitals. Although microaerophilic conditions were achieved in a candle jar for bacterial incubation on chocolate agar at $37 \pm 2^{\circ}C$ for 24 to 48 hours, blood agar and MacConkey agar were incubated in aerobic conditions at $37 \pm 2^{\circ}C$ for 18 to 24 hours.[27,28] Oxoid™ Microbact™ 12A/12B (Oxoid Limited, Basingstoke, Hampshire, UK), a microplate-based biochemical test, was used to identify gram-negative bacteria, and the conventional method according to Bergey's system of classification.[28–30] An automated VITEK®2 system (BioMérieux, Marcy-l'Étoile, France) was used for bacterial identification at Atma Jaya Hospital.[31] Nucleic acid extraction was conducted for all specimens that underwent bacterial cultivation without waiting for the cultivation result.

## Bacterial identification

PneumoCLART bacteria® is an emerging molecular diagnostic technique with immense potential for the detection of human respiratory pathogens. In brief, the PneumoCLART bacteria® detection system is based on the precipitation of an insoluble product in areas on the microarray where the hybridization of amplification products with specific probes occurs. During PCR, amplified products are labeled with biotin. After amplification, these biotin-labeled products are hybridized with their respective specific complementary probes, which are then immobilized on specific known microarrays. Next, these probes are incubated with a streptavidin–peroxidase conjugate, which is bound to their specific probes. The peroxidase activity induces the appearance of a non-soluble product in the presence of the substrate *o*-dianisidine, which precipitates in the areas on the microarray where hybridization occurs. Finally, the precipitation of the substrate and control is read by the CAR® clinical array reader, which generates an objective clinical report (www.genomica.com).

In this study, the molecular analysis was performed using PneumoCLART bacteria®, which facilitated the *in vitro* detection and characterization of bacteria in the respiratory samples. Total nucleic acids were extracted from each specimen using an automated NUCLISENS® easyMag® system (BioMérieux) according to the manufacturer's instructions, followed by amplification reaction and visualization of the amplified product in the CLART® strip (www.genomica.com). The amplification tubes had primers specific for a range of bacteria with established roles in respiratory tract infections including *M. pneumoniae*, *S. aureus*, *S. pneumoniae*, *Chlamydia pneumoniae*, *Moraxella catarrhalis*, *Haemophilus*, and *Bordetella* spp. (*B. pertussis*, *B. parapertusis*, *B. bronchiseptica*, *B. holmesii*). Endogenous genomic DNA and internal controls were included to ensure the efficiency of the assay and to eliminate false negatives. This examination was performed at Sentra Diagnostica Dinamika, a private laboratory in Jakarta, Indonesia. The laboratory staff involved in

this study was unaware of the independent test results.

## Results

This study enrolled 27 adults, including 11 women and 16 men aged 35 to 90 years, with pneumonia requiring hospitalization at Atma Jaya Hospital. Specifically, we sought patients who could expel active sputum spontaneously, and bacterial cultivation was performed.

The quality of the collected sputum was assessed, and the saliva-contaminated specimens accounted for almost 30% of the total number of samples (8/27) with SEC counts >10/low-power field (LPF). Our laboratory defines the adequacy of any specimen for the lower respiratory tract as an area containing SEC < 10/LPF and polymorphonuclear neutrophil or leukocyte counts ≥25/LPF.[32,33] Accordingly, 15 representative specimens (55%) fulfilled the aforementioned criteria.

Gram staining performed before cultivation revealed the presence of various gram-negative and gram-positive organisms in the specimens. Accordingly, 39 organisms were isolated from 27 sputum specimens. Among these, *Klebsiella pneumoniae* (12.8%), yeast (12.8%), and viridans group *Streptococci* were present in abundance (12.8%, Figure 1).

According to the PneumoCLART bacteria® examination, 7 of 27 patients with pneumonia were positive for *M. pneumoniae* (26%). Among these, only two of the seven specimens positive for *M. pneumoniae* were adequate for analysis (Table 1). Notably, 50% of patients positive for *M. pneumoniae* required admission to the intensive care unit (ICU) at Atma Jaya Hospital (Figure 2).

Briefly, the time of disease onset in these patients ranged from less than 8 hours to 30

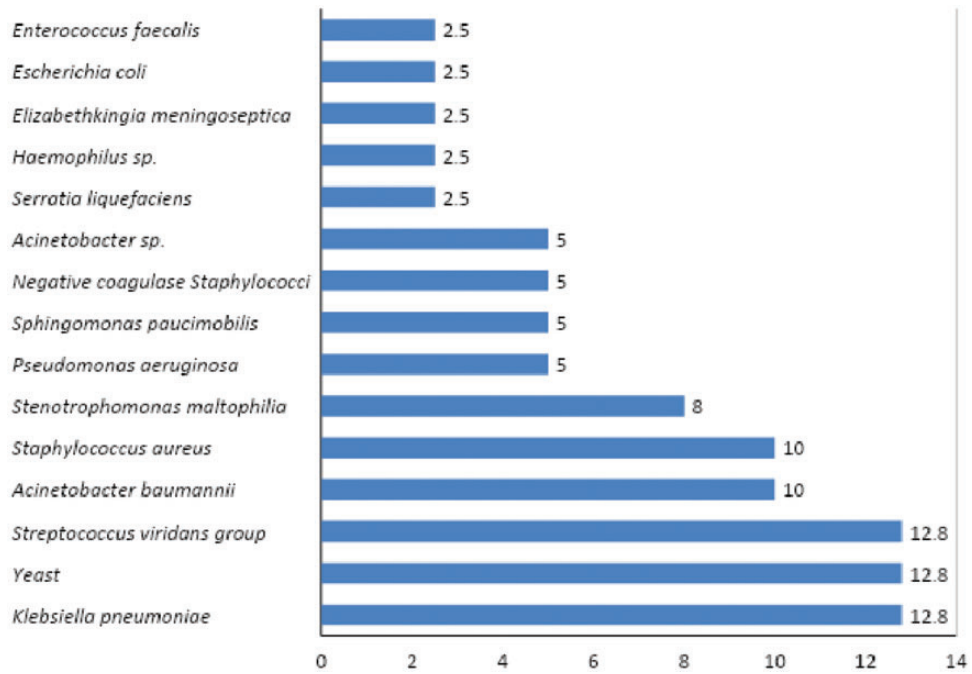


**Figure 1.** Percentage of each isolate cultivated from sputum specimens (n = 39).

**Table 1.** Aerobic bacterial cultivation result and microscopic characteristics of the sputum from the *Mycoplasma pneumoniae*-positive group.

| Age (years) | Organism | Saliva contamination | Inflammation parameter | Location/origin |
|---|---|---|---|---|
| 42 | *K. pneumoniae*, *P. aeruginosa* | + | − | ICU |
| 67 | *Haemophilus* sp. | + | + | General ward |
| 37 | *S. maltophilia*, *S. viridans* | + | + | General ward |
| 48 | Yeast | − | + | General ward |
| 40 | *K. pneumoniae* | + | + | General ward |
| 74 | *A. baumannii*, *S. maltophilia* | − | + | ICU |
| 75 | Yeast | − | − | ICU |

ICU, intensive care unit.

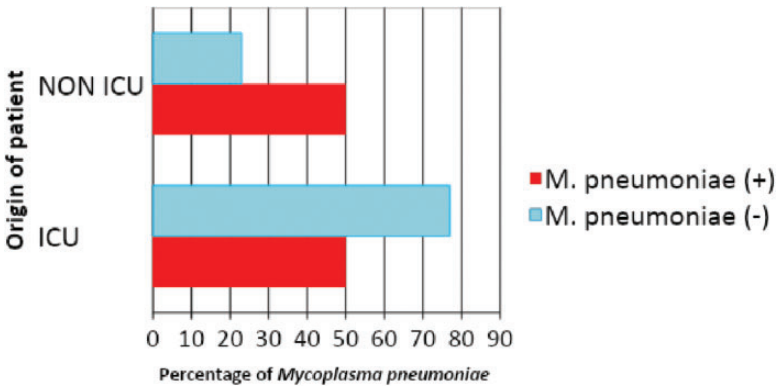


**Figure 2.** Result of *Mycoplasma pneumoniae* detection by PneumoCLART bacteria®.

days. Among these, most of the patients received antibiotic treatment regimens comprising doripenem, levofloxacin, azithromycin, cefadroxil, levofloxacin, vancomycin, and meropenem. Further, the comorbidities in these patients included cardiovascular disease, diabetes mellitus (DM), hypertension, benign prostatic hyperplasia (BPH), chronic kidney disease (CKD), nucleus pulposus herniation, and scoliosis. Furthermore, the imaging features included pleural effusion, lung infiltrates, pleuropneumonia, bronchitis, scoliosis thoracalis, interposition colon, interstitial pneumonia, traction bronchiectasis, lower lobe atelectasis, bronchopneumonia, and pneumothorax. The clinical manifestations included dyspnea, hematemesis, necrotizing fasciitis, CKD, hyperkalemia, type II DM, melena, dehydration-associated hypotension, BPH, nucleus pulposus herniation, and suspected sepsis (attributable to elevated procalcitonin levels). Most importantly, normocytic normochromic anemia, anemia of chronic disease (ACD), ACD with infection anemia, thrombocytopenia, leukocytosis, infection or liver disease, and autoimmune disorder were also suspected in two patients who tested positive for infection by *M. pneumoniae* using PneumoCLART bacteria®.

## Discussion

There are accumulating data on the high prevalence of *M. pneumoniae* as a major

culprit causing CAP globally. In a prospective study, Liu *et al*. reported *M*. *pneumoniae* as the most prevalent pathogen (20.7%, 126/610) among urban Chinese adults with CAP.[34] Further, Wu *et al*. reported the highest detection rate of 56.9% (among 10,435 specimens) for *M*. *pneumoniae* among all pathogens tested in Chinese pediatric patients (age <16 years) with acute respiratory infections.[35] Similarly, Chen *et al*. identified *M*. *pneumoniae* as the predominant pathogen (positivity rate = 40.78%) among all tested pathogens in Chinese pediatric patients (aged 4–14 years).[36] Furthermore, Chen *et al*. reported a high *M*. *pneumoniae* prevalence (55%) in the pediatric population using an IgM antibody-based immune-gold labeling detection method.[37] In a retrospective study, *M*. *pneumoniae* was the most dominant causative agent in 14.5% of pediatric CAP cases (166 pneumonia-confirmed cases; aged 1–15 years) in Belgrade, Serbia.[38] Another prospective study identified *M*. *pneumoniae* infection among 27% of pediatric patients (140 children aged 2 months to 15 years) with CAP in Istanbul, Turkey.[39] In a cross-sectional study, Carcey *et al*. recorded a high positivity of 31.9% for *M*. *pneumoniae* in 20,020 serological samples from Chilean children using IgM serological testing (age <18 years).[40] In their consecutive cross-sectional study, Del *et al*. disclosed a high prevalence (25.19% or 170/675 pediatric patients) of *M*. *pneumoniae*-associated acute respiratory infections in Peruvian children (age <18 years).[41]

Another retrospective study reported *M*. *pneumoniae* as a possible pathogen in Chinese patients (3852 adults and 3983 children) with respiratory tract infections admitted to the ICU during the epidemic (2011–2013). The positivity rate for *M*. *pneumoniae* was 21.2% with no statistically significant difference noted among different age groups during most of the epidemic.[42] In a 5-month study (from January 2017 to June 2017), Arfaatabar *et al*. observed a high frequency of *M*. *pneumoniae* among 520 patients with CAP in Tehran, Iran.[43] Su *et al*. determined that *M*. *pneumoniae* was highly prevalent in hospitalized children with community-acquired *M*. *pneumoniae* pneumonia (MPP) (66.4% [221/333 pediatric patients]).[44] A retrospective study by Cheng *et al*. investigated the epidemiology of *M*. *pneumoniae* in Chinese children with respiratory infections from June 2016 to May 2021.[45] Reportedly, the positivity rates did not differ significantly in relation to the season, age group, gender, or period (before or during the COVID-19 pandemic) as revealed from the *M*. *pneumoniae* specific-IgM antibody rapid immunochromatographic assay of the serum specimens of 569,887 pediatric patients.[45]

Treatment strategies for CAP largely rely on the presented clinical symptoms (mild or severe) and infection type (bacterial- and/or viral-associated pneumonia; co-infection) suspected in patients with CAP.[4,46–48] Over time, there has been a change in the CAP etiology concerning bacterial, viral, and fungal co-infections. It is therefore recommended to initially administer empiric antibiotic treatment to eradicate the major causative pathogens and resolve clinical symptoms.[46–49] Precisely, the pre-hospitalization treatment regimen comprises oral macrolides (for example, azithromycin or clarithromycin and erythromycin), tetracycline (for example, doxycycline or vibramycin), and fluoroquinolones (for example, ciprofloxacin and levofloxacin) for managing CAP-associated clinical symptoms in patients. Alternatively, oral administration of amoxicillin or clavulanate and $\beta$-lactams (*e.g*., cefpodoxime, cefprozil, cefuroxime) can relieve mild symptoms in patients with no co-morbidities (*e.g*., renal failure, chronic obstructive pulmonary disease, diabetes, asplenia, congestive heart failure, chronic alcoholism, immunosuppressive

conditions).[46–48] However, clinical guidelines restrict the use of macrolides in patients with community-acquired MPP given the growing concerns for resistance in this pathogen.[12,50–54] Moreover, $\beta$-lactam antibiotics are ineffective against *M. pneumoniae* because it lacks a rigid cell wall.[47,48] Further, this study was undertaken to assess the diagnostic efficiency of PneumoCLART bacteria® regarding *M. pneumoniae*, but this study was not designed to recommend pre-hospitalization treatment for *M. pneumoniae*-associated CAP. The antibiotic treatment administered to the patients after admission to Atma Jaya Hospital was recorded.

*M. pneumonia*, as a fastidious pathogen, presents several challenges concerning its efficient and accurate diagnosis.[16] Of the seven samples positive for *M. pneumoniae*, two expectorated sputum specimens featured a leukocyte count of <25/LPF. In addition, 50% of the sputum specimens that failed to fulfill the criteria for adequacy were either contaminated with saliva or obtained from patients who were not admitted to the ICU. Further, one-fourth of specimens negative for *M. pneumoniae* featured a leukocyte count of <25/LPF. Among negative samples, fewer than 25% that failed to fulfill the criteria for adequacy were contaminated with saliva or obtained from patients not admitted to the ICU (Table 2). PneumoCLART bacteria® could be a useful diagnostic technique for detecting *M. pneumoniae* in patients with respiratory illnesses, especially community-acquired MPP. However, further studies including an appropriate number of specimens with stringent quality control

**Table 2.** Aerobic bacterial cultivation result and microscopic characteristics of sputum from the *Mycoplasma pneumoniae*-negative group.

| Age (years) | Organism | Saliva contamination* | Inflammation parameter# | Location/origin |
|---|---|---|---|---|
| 80 | *S. liquefaciens* | + | − | ICU |
| 72 | *A. baumannii* | + | − | General ward |
| 46 | Yeast | + | + | ICU |
| 65 | *S. viridans* | − | + | General ward |
| 45 | *Acinetobacter* sp. | + | + | General ward |
| 85 | *S. gordonii*, *S. paucimobilis* | − | + | IMC |
| 62 | *A. lwoffii*, *S. haemolyticus*, *S. parasanguinis* | − | + | IMC |
| 77 | *S. aureus*, *P. aeruginosa* | − | + | General ward |
| 52 | *A. baumannii*, *E. meningoseptica* | − | − | ICU |
| 67 | *S. haemolyticus* | − | + | ICU |
| 62 | *A. baumannii* | − | + | ICU |
| 88 | *S. aureus* | − | + | IMC |
| 54 | *S. paucimobilis* | − | + | ICU |
| 42 | *K. pneumoniae* | − | − | ICU |
| 72 | *S. aureus* | − | + | ICU |
| 76 | *E. coli* | − | + | IMC |
| 90 | *K. pneumoniae* | − | + | General ward |
| 86 | *S. pseudoporcinus*, *E. faecalis*, *S. aureus* | − | − | IMC |
| 52 | Yeast | − | + | ICU |
| 80 | Yeast | − | + | IMC |

*Squamous epithelial cell count >10/low power field (LPF); #polymorphonuclear neutrophil count ≥25/ LPF.
ICU, intensive care unit; IMC, intermediate care unit.

measures for collection and analysis will be indispensable for strengthening the utility of this technique. The small sample size was a limitation of the present study.

## Conclusion

In our study, some samples featured a low leukocyte count and/or contamination by saliva; hence, the findings on the utility of CLART® microarray technology for the identification of *M. pneumoniae* in pneumonia-positive patients need further validation. Moreover, we speculate that the small sample size might have affected the outcomes of this prospective study. In conclusion, molecular diagnostic methods based on the amplification of nucleic acids (DNA/RNA) such as CLART® microarray technology might improve the sensitivity for *M. pneumoniae* identification provided a sufficient sample size is obtained and no samples are contaminated.

### Acknowledgements

Experimental support from the Director and committee of Atma Jaya Hospital and Dr. Lely Saptawati of Dr. Moewardi Hospital (Surakarta, Central Jawa, Indonesia) is acknowledged.

### Author contributions

ET, LHM, and SJ: Conceptualization, visualization, methodology, validation, data analysis and curation, original draft preparation, and review. LL and FP: Methodology, review, and editing. All authors contributed significantly to the study and approved the final manuscript.

### Data availability

All relevant data are presented in the manuscript, and the data are accessible in the peer-review process.

### Declaration of conflicting interests

The authors have no conflicts of interest to disclose.

### Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: We thank the School of Medicine, Atma Jaya Catholic University of Indonesia (Jakarta, Indonesia) for providing financial support (funding number: 0831/III/D.FM-PM.10.01/04/2018) for this study.

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