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RESEARCH ARTICLE

Staphylococcus aureus and *Pseudomonas aeruginosa* in Tubotympanic Chronic Suppurative Otitis Media Patients in Purwokerto, Indonesia

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Abstract

BACKGROUND: Chronic Suppurative Otitis Media (CSOM) causes hearing impairment and frequently occurred in low-income country where medical care and personal hygiene are poor. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most common cause of CSOM. We investigated prevalence and antimicrobial susceptibility of *S. aureus* and *P. aeruginosa* from tubotympanic CSOM patients in tertiary hospital, Purwokerto, Indonesia in 2016-2017.

METHODS: Ear swab specimens were collected from patients with tubotympanic CSOM. *S. aureus* and *P. aeruginosa* were isolated and identified by culture, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), and molecular tools. Antimicrobial susceptibility testing was performed using the disk diffusion method.

RESULTS: Out of ear swabs from 34 patients with tubotympanic CSOM, *P. aeruginosa* and *S. aureus* were identified in 35% patients. No Methicillin-resistant *S. aureus* (MRSA) strain was found from the ear swabs of the patients with tubotympanic CSOM. Bacterial identification using the MALDI-TOF MS was concordantly with culture and molecular tools. All *S. aureus* isolates showed full susceptibility to cefoxitin and trimethoprim-sulphamethoxazole. Resistance to tetracycline was common with only 64% of *S. aureus* strains being susceptible. Meanwhile, all *P. aeruginosa* strains were susceptible to cefepime, cetazidime, meropenem, gentamicin, and tobramycin.

CONCLUSION: *S. aureus* and *P. aeruginosa* are found in patients with tubotympanic CSOM and still susceptible to different antibiotic agents. MALDI-TOF MS demonstrate rapid, accurate and robust to detect *S. aureus* and *P. aeruginosa*.

KEYWORDS: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, chronic tubotympanic suppurative otitis media

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Introduction

Chronic suppurative otitis media (CSOM) is defined as a long-term inflammation of the middle ear and mastoid cavity accompanied by purulent discharge and tympanic perforation for more than 6 weeks. The disease remains one of the most common childhood diseases.(1) Tubotympanic and atticoantral diseases are two categories of CSOM.(2) Tubotympanic CSOM rarely causes serious complication

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and not correlated to cholesteatoma.(3) CSOM showed a prevailing and notorious infection in low-income countries, causes serious local damage and threatening complications.(4) Chronic suppurative otitis media were found among 67% Indonesian children 6-15 years of age and contributed to 57% of hearing loss.(5) Otitis media related hearing loss were significantly higher in rural than urban area.(5)

Tubotympanic CSOM possibly occurred due to infection caused by viral or bacteria, or factors related to allergy, immunology, environment and socioeconomic status.(2) Bacterial colonization in nasopharynx can enters the middle ear and causes infection. *Pseudomonas aeruginosa* followed by *Staphylococcus aureus* is the most common cause of CSOM.(4)

P. aeruginosa is one of frequent bacteria causing healthcare-associated infections especially in patients with critical underlying medical conditions. However, treatment on infection caused by *P. aeruginosa* is hard due to its resistance to antibiotics and the ability in biofilm formation. The infections will lead to mortality of the patients.(6) Many previous studies reported the presence of *P. aeruginosa* infection cases and its resistance to antibiotics. A study in Angola reported that there were 13% of *P. Aeruginosa* identified from the ear discharge of CSOM patients.(7) Meanwhile, 32% of *S. aureus* and 31% of *P. aeruginosa* were detected from CSOM patients in a tertiary care hospital in India.(8)

In Indonesia, 70% of children suffered from CSOM.(5) A recent study discovered Mannose-Binding Lectin (MBL) deficiency in 22% tubotypmpanic CSOM patients in Purwokerto, Indonesia.(9) However, the pathogen responsible for CSOM disease remains unclear in Indonesia. Aerobic, anaerobic bacteria, and fungi are possible causes of CSOM, which can be together to cause infection. *P. aeruginosa*, *S. aureus*, and *Proteus* sp. are causative agent of CSOM from aerobic bacteria group.(7) Whereas, anaerobic bacteria such as Clostridium, Peptococcus, Peptostreptococcus, Prevotella, and Bacteriodes species are also frequently causes CSOM infection. Meanwhile *Aspergillus niger*, *Aspergillus fumigatus*, *Candida* spp., were the most common fungi to cause CSOM infection.(10)

Epidemiological data of *S. aureus* and *P. aeruginosa* from CSOM patients in Indonesia are limited. In this study, we investigate the prevalence and antimicrobial susceptibility of *S. aureus* and *P. aeruginosa* among tubotympanic CSOM patients in a tertiary hospital, Purwokerto, Indonesia in 2016-2017.

Methods

Study Design

A cross-sectional study was conducted in the Otorhinolaryngology Clinic at RSUD Prof. Dr. Margono Soekarjo Hospital, Purwokerto, Indonesia, from September 2016 to February 2017. The study was approved by the Research Ethics Committee at the Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto, Indonesia (No. 137/KEPK/VI/2016).

Sample Collection

Ear swab specimens were collected unilaterally (one ear) or bilaterally (both ears) from CSOM patients who met inclusion criteria according to Indonesian Association of Otorhinolaryngology/Perhimpunan Ahli Ilmu Penyakit THT Indonesia (PERHATI) guidelines. Obtained ear swabs were placed in Skim milk, Tryptone, Glucose, and Glycerol (STGG) transport media and stored at 4°C for maximum 6 hours then stored at -80°C in Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia.(11) The ear swabs in STGG were sent to Molecular Bacteriology Unit, Eijkman Institute for Molecular Biology, Jakarta for bacteriology analysis.

Isolation and Identification of *S. aureus*

Isolation and identification of *S. aureus* was performed at Molecular Bacteriology Unit, Eijkman Institute for Molecular Biology, Jakarta. Ear swab-inoculated STGG media was transferred for 200 µL into enrichment media consisted of 5.0 ml Todd Hewitt broth with 0.5 % yeast extract and 1 mL rabbit serum, then incubated at 35-37°C for 5 hours.(12) Ten microliters of enriched broth was plated onto Mannitol Salt Agar (MSA) and Mac Conkey Agar followed by incubation at 35-37°C for 20 hours. *S. aureus* was defined as a yellow colony on MSA agar while *P. aeruginosa* was characterized as a colorless colony on Mac Conkey agar. Single colony of suspected *S. aureus* was subcultured onto sheep blood (5%) agar to observe beta-hemolytic resembling *S. aureus*. Meanwhile suspected *P. aeruginosa* was subcultured on Kligler Iron Agar for further identification. The identification for suspected *S. aureus* was continued to Gram staining, catalase, oxidase and coagulase test, while suspected *P. aeruginosa* was tested only for Gram staining, catalase, and oxidase. The presumptive isolates *S. aureus* was defined as a colony with beta-hemolysis, Gram positive with grape-like formation, catalase positive, coagulase positive, and oxidase-negative.(13) Meanwhile,

presumptive isolates of *P. aeruginosa* was characterized as a non-glucose and non-lactose-fermenting Gram negative bacteria with a rod shape, catalase and oxidase positive.(14)

Microflex LT Matrix-Assisted Laser Desorption Ionization – Time of Flight Mass Spectrophotometry (MALDI-TOF MS) identification

Bacterial isolates were identified using MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) equipped with flex control software (Bruker Daltonics) as previously described.(15) A direct colony smear (DCS) on a ground plate was prepared by spreading a single colony from an overnight culture then air-dried at room temperature. After that, 1 µL of matrix solution (α-cyano-4-hydroxycinnamic acid) (Bruker Daltonics) was added and dried at room temperature. To obtain spectra, the MALDI-TOF was set at linear positive-ion, voltage at 20kV, and 2-20 kDa as a mass range. Results in numeric score were ranged from 0 until 3.000 according to the similarity estimation of measured protein spectra with protein database in MALDI-Bruker's Biotyper-specific software. Score below 1.69 was classified as unreliable identification, 1.70-1.99 as a probable genus identification, secure genus identification for 2.00-2.29 score, and 2.30-3.00 as a highly probable species.(15,16)

Molecular Identification of *S. aureus* and Methicillin Resistant *S. aureus* (MRSA)

DNA extraction was performed using Boil method as previously described.(15) Duplex Polymerase Chain Reaction (PCR) was performed to confirm *S. aureus* and to identify MRSA. A 255 bp gene encoding nuclease (*nuc*) was detected for internal control of *S. aureus* with forward primer (5'-TCAGCAAATGCATCACAAACAG-3) and reverse primer (5'-CGTAAATGCACTTGCTTCAGG-3'). Whereas, Methicillin Resistant *S. aureus* (MRSA) were identified by detecting presence of mecA gene resulting 527 bp amplicon with forward primer (5'-GGGATCATAGCGTCATTATTC-3') and reverse primer (5'-AACGATTGTGACACGATAGCC-3'). The PCR was performed under following conditions: 94°C for 5 minutes followed by 35 cycles of at 94°C for 30 sec, annealing at 55 for 30 sec, and elongation at 72 for 1 minute. The last stage was heated at 72°C for 10 minutes. DNA band was visualized using gel electrophoresis with 2% agarose and 100V for 90 minutes.(16)

16S rRNA sequencing of *P. aeruginosa*

The 16S ribosomal DNA(rRNA) provide species-specific sequence and stable genetic code to identify *P. aeruginosa.* (17) Briefly, 16S sequencing was initially done by generating amplicon of 16S rRNA region using 8F as forward primer (5'-AGAGTTTGATCCTGGCTCAG-3'), and 1492R as reverse primer (5'- GGTTACCTTGTTACGACTT-3'). PCR amplification was performed under following conditions: 94°C for 3 minutes followed by 30 cycles of 94°C for 1 minute, 48°C for 1 minute, 74°C for 1 minute, and 72°C for 10 minutes. Amplicon was continued for cycle sequencing as follows: 96°C for 3 minutes then 35 cycles of 96°C for 10 sec, 50°C for 5 minutes, and 60°C for 4 minutes.(18)

Antimicrobial Susceptibility Testing

Antibiotic susceptibility test using disk diffsuion was employed among 12 *P. aeruginosa* isolates. Bacterial suspension equivalent to a 0.5 MacFarland standard was made from fresh and pure colonies in 5 mL of saline 0.85%. Cotton swab was dipped into bacterial suspension then pressed gently on the wall of glass tube. After that, cotton swab was streaked onto Mueller Hinton Agar and incubated in the ambient air at 37°C for 16-20 hours. Antibiotics used for *P. aeruginosa* testing were 30 µg cefepime, 30 µg ceftazidime, 30 µg aztreonam, 10 µg meropenem, 10 µg gentamicin, 10 µg tobramycin, 5 µg and ciprofloxacin. Meanwhile, tested antibiotics for *S. aureus* susceptibility were 2 µg clindamycin, 30 µg chloramphenicol, 1 µg oxacillin, 30 µg tetracycline, 15 µg erythromycin, 1.25 µg trimethophrim/23.75 µg sulfamethoxazole, 30 µg cefoxitin, and 10 µg gentamicin. Interpretation of clear zone was followed the guidelines from Clinical Laboratory Standard Institute (CLSI) 2019.(18) Cefoxitin was used to assess methicillin resistance. Isolates with mecA positive and cefoxitin resistant were defined as methicillin resistant.(20)

Data Analysis

Chi-square was performed to assess contribution of risk factors to *S. aureus* and *P. earuginosa* infection in tubotympanic CSOM. If the expected cells counted less than 5, Fisher test was used to determine risk factors that may associate to the infection of *S. aureus* and *P. aeruginosa* in CSOM disease. A p-value<0.05 considered as statistically significant. Potential risk factors shown in Table 1 were tested by Mantel Haenszel to calculate common Odd Ratio.

Results

A total of 34 patients with tubotympanic CSOM were enrolled during the study period and 40 ear swabs were obtained since there were 6 patients whom collected

Table 1. Characteristics of patients with chronic tubotympanic suppurative otitis media in Purwokerto, Indonesia.

Characteristics	n	Patients Infected by *S. aureus* n (%)	Odd Ratio (OR)	*p*-value	Patients Infected by *P. aeruginosa* n (%)	Odd Ratio (OR)	*p*-value
Age							
12-24 years old	11	2 (6)	3.460	0.252	2 (6)	0.530	0.459
>24 years old	23	10 (29)			7 (21)		
Sex							
Female	22	8 (24)	0.880	1.000	6 (18)	2.670	0.265
Male	12	4 (12)			6 (18)		
Education background							
Elementary school	8	2 (6)	1.880	0.681	3 (9)	0.880	1.000
Others	26	10 (29)			9 (26)		
Employment							
Student	7	2 (6)	1.480	1.000	4 (12)	0.180	0.211
Others	27	10 (29)			8 (24)		
Marriage status							
Married	20	10 (29)	6.000	0.066	5 (25)	0.330	0.163
Not Married	14	2 (6)			7 (50)		

bilaterally. Patients older than 24 years were more likely to be infected by *S. aureus* (OR=3.460; *p*-value=0.252) and *P. aeruginosa* (OR=0.530; *p*-value=0.459). In addition, married ones have more risk tosuffer from otitis media caused by *S. aureus* (OR=6.000; *p*-value=0.066), but *P. aeruginosa* infection was more occurred among not married patients (OR=0.330; *p*-value=0.163). However, there were no significant differences within age, sex, educational background, employment or marital status that contributing to *S. aureus* and *P. aeruginosa* infection in tubotympanic CSOM (Table 1). Fourteen *S. aureus* isolates were obtained from 40 ear swabs of CSOM patients (35%). The prevalence of *S. aureus* was 35 % (12/34) among patients with tubotympanic CSOM since two patients who were collected ear swabs bilaterally, showed *S. aureus* positivity for both of ears. MALDI-TOF MS was performed to confirm presumptive isolates as described before (n=30) (Table 2). Among *S. aureus* isolates (14/30), 50% (7/14) were identified as highly probable species ID (score 2.30-3.00) and 50% (7/14) were grouped into secure genus ID (score 2.00-2.29). However, there were 10% (3/30) isolates that could not be determined by MALDI-TOF MS. Meanwhile, 43% (13/30) isolates were identified as other species of Staphylococcus using MALDI-TOF MS such as *S. capitis* (n=1), *S. warneri* (n=1), *S. caprae* (n=3), *S. haemolyticus* (n=3), *S. sciuri* (n=1), *S. epidermidis* (n=3), *S. cohnii* (n=1). Duplex PCR was performed to detect nuc and mecA genes among confirmed 14 *S. aureus* isolates (Figure 1). All of the *S. aureus* isolates were positive for *nuc* gene (Table 2). In this study, we did not found any Methicillin Resistant *S. aureus* (MRSA) since all of the *S. aureus* isolates were negative for *mecA* gene. The identification of *S. aureus* was 100% match between Duplex PCR (*nuc* and *mecA*) and MALDI-TOF MS.

Thirty percent (12/40) of ear swabs of tubotympanic CSOM patients were positive for *P. aeruginosa*. The prevalence of *P. aeruginosa* isolated from tubotympanic CSOM patients was 35% (12/34). Obtained presumptive *P. aeruginosa* isolates (22/34) were identified by conventional

Table 2. Identification of *Staphylococus aureus* strains collected from patients with chronic tubotympanic suppurative otitis media in Purwokerto, Indonesia.

S. aureus Identification	n (%)
Presumptive isolate	30 (100)
MALDI-TOF MS Biotyper identification	
2.30-3.00[a]	7 (23)
2.00-2.29[b]	7 (23)
1.70-1.99[c]	0
<1.69[d]	0
Not identified	3 (10)
Other bacteria[e]	13 (43)
PCR-positive for *nuc* gene[f]	14 (47)
PCR-positive *mecA* (MRSA) gene	0

[a]Highly probable; [b]Secure; [c]Probable; [d]Non-reliable; [e]ID score >1.8 as *S. capitis* (1), *S. warneri* (1), *S. caprae* (3), *S. haemolyticus* (3), *S. sciuri* (1), *S. epidermidis* (3), *S. cohnii* (1); [f]Two patients who were collected ear swabs bilaterally, showed *S. aureus* positivity at both ears.

Table 3. Identification of *Pseudomonas aeruginosa* strains collected from patients with chronic tubotympanic suppurative otitis media in Purwokerto, Indonesia.

P. aeruginosa Identification	n (%)
Presumptive isolate	22
MALDI-TOF MS Biotyper identification	
2.30-3.00[a]	11 (50)
2.00-2.29[b]	1 (5)
1.70-1.99[c]	0
<1.69[d]	0
Others[e]	10 (45)
16S rRNA sequencing result	12 (55)

[a]Highly probable; [b]Secure; [c]Probable; [d]Non-reliable; [e]ID Score >2 as *Providencia stuartii* (4), *Escherichia coli* (2), *Achromobacter xylosoxidans* (1), *Enterobacter asburaie* (1), *Proteus mirabilis* (1), *Acinetobacter baumanii* (1).

microbiology technique. Half of the of *P. aeruginosa* presumptive isolates (11/22) were determined as a highly probable species ID (score 2.30-3.00) by MALDI-TOF MS and one isolate (1/22) was secure genus ID of *P. aeruginosa* (Table 3). Fourty-five percent (10/22) of presumptive isolates were identified using MALDI-TOF MS as *Providencia stuartii* (n=4), *Escherichia coli* (n=2), *Achromobacter xylosoxidans* (n=1), *Enterobacter asburaie* (n=1), *Proteus mirabilis* (n=1), and *Acinetobacter baumanii* (n=1). The 16S rRNA sequencing was performed to confirm MALDI-TOF MS results. PCR amplification of 12 *P. aeruginosa* isolates identified by MALDI-TOF MS showed 1492 bp positive of 16S rRNA gene (Figure 2). Moreover, 16S rRNA sequencing confirm all of the identified *P. aeruginosa* by MALDI-TOF MS were *P. aeruginosa* (Table 6). Thus, all of the P. aeruginosa isolates identified with MALDI-TOF MS were concordance with culture and 16S rRNA sequencing results.

The majority of *S. aureus* isolates were susceptible to tested antibiotics. All of the isolates showed full susceptibility to cefoxitin and trimethoprim/sulfamethoxazole. Whereas, resistance level to tetracycline was 36%, while non-susceptible isolates to clindamycin and oxacillin were 21%. Most of the *S. aureus* isolates still suscpetible to gentamicin 93%. (Table 4). One *S. aureus* isolate was resistant to more than 3 classes of antibiotics or classified as multidrug resistant (MDR) *S. aureus*. However, we did not found MRSA since all of the *S. aureus* isolates were still susceptible to cefoxitin. Meanwhile, no resistance detected against cefepime, ceftazidime, meropenem, gentamcin, and tobramycin, among *P. aeruginosa* isolated from CSOM patients. However, *P. aeruginosa* isolates demonstrated intermediate susceptibility to aztreonam (8%) and ciprofloxacin (17%) (Table 5).

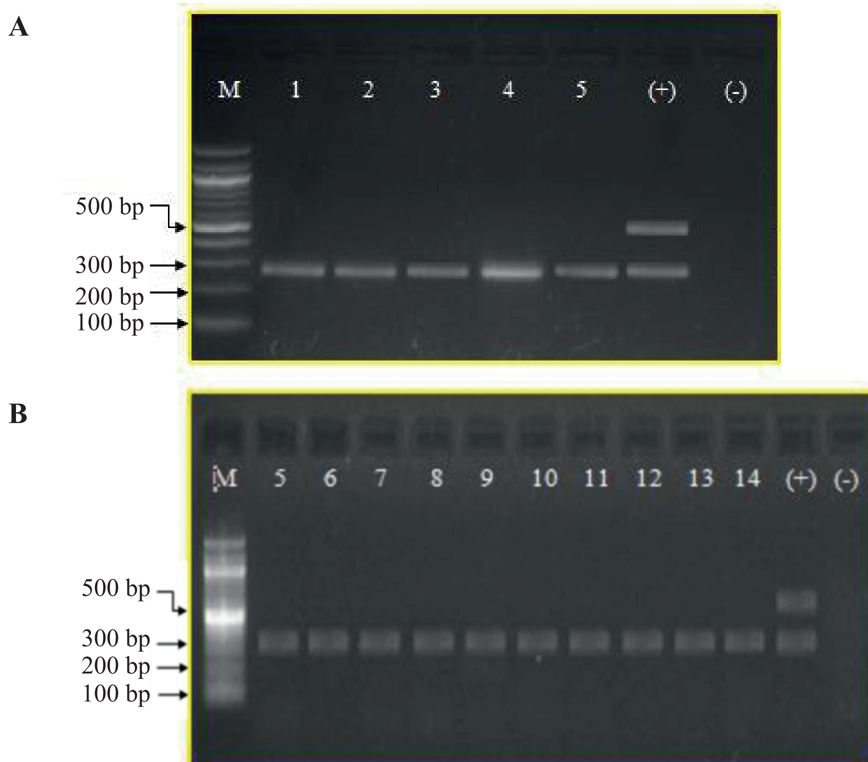

Figure 1. Duplex PCR of *nuc* and *mecA* genes amplifications results from DNA of 14 *S. aureus* isolates. A: A detection of 255 bp *nuc* gene using PCR; B: Gel Electrophoresis of 528 bp *mecA* gene amplification. Line M is is a DNA marker 100-1500 bp. Line 1-14 are the DNA of 14 *S. aureus* isolates from this study. Line (+) is positive control of *nuc* and *mecA* genes. Line (-) is a negative control.

Table 4. Antimicrobial susceptibility of *S. aureus* isolates.

Antimicrobial Agent	Number of Susceptibility (%)		
	Susceptible	Intermediate	Resistant
Cefoxitin	14 (100)	0	0
Penicillin	12 (86)	0	2 (14)
Gentamicin	13 (93)	0	1 (7)
Erythromycin	12 (86)	1 (7)	1 (7)
Tetracycline	9 (64)	0	5 (36)
Trimethoprim/sulfamethoxazole	14 (100)	0	0
Chloramphenicol	12 (86)	0	2 (14)
Clindamycin	11 (79)	1 (7)	2 (14)

Discussion

Prevalence of *P. aeruginosa* and *S. aureus* from patients with CSOM was the same (35%). Our finding was similar with previous study in India that *P. aeruginosa* (32%) and *S. aureus* (30%) were dominant bacterial species isolated from patients with CSOM infection. Interestingly, in the present study, there were 4 patients infected with both by *S. aureus* and *P. aeruginosa*. This might be a mutualistic association between *S. aureus* and *P. aeruginosa* causing CSOM infection. Previously, synergetic interactions were recorded between *S. aureus* and *P. aeruginosa* in cystic fibrosis and wound. The interaction causes biofilm formation and enhances antimicrobial resistance.(21) However, both of *P. aeruginosa* and *S. aureus* were still susceptible to most of the used antibiotics in this study.

P. aeruginosa and *S. aureus* travel from the external canal and reach the middle ear to initiate CSOM infection. Pathogenesis of *P. aeruginosa* involves several virulence factors such as adhesion, toxin, exoenzyme, alginate and biofilm.(19) While *S. aureus* has the ability to invade human middle ear epithelial cells through cholesterol-dependent pathway and causes chronic suppurative otitis media.(22) The majority of *P. aeruginosa* isolates were still susceptible to tested antibiotics (cefepime, ceftazidime, meropenem, gentamcin, tobramycin, aztreonam, and ciprofloxacin). No carbapenem-resistant *P. aeruginosa* (CRPA) detected in this study. This finding is in line with the study in India that most of the of *P. aeruginosa* from CSOM patients were still susceptible to aminoglycoside (gentamicin, tobramicin), cephalosporin (ceftazidime, cefepime), antipseudomonal (piperacillin), piperacillin/tazobactam, and carbapenem.(23) Chloramphenicol, aminoglycoside, and quinolone were the most frequently used antibiotics drop to treat tubotympanic CSOM infection in Indonesia (24). A high level of susceptible *P. aerugionsa* in this study might be caused by rare exposure to antibiotics as a treatment in CSOM infection. However, susceptible *P. aeruginosa* are significantly more virulent compared to Multidrug Resistant *P. aeruginosa*, and potentially causes serious infection in future. Susceptible *P. aeruginosa* has a blue-green color that indicates pyocanin pigment, one of the *P. aeruginosa* important factors. Meanwhile, MDR strains appear as yellowish-green color and produce significantly less amount of pyocyanin.(25,26)

Table 5. Antimicrobial susceptibility of *P. aeruginosa* isolates.

Antimicrobial Agent	Number of Susceptibility (%)		
	Susceptible	Intermediate	Resistant
Cefepime	12 (100)	0	0
Ceftazidime	12 (100)	0	0
Aztreonam	11 (92)	1 (8)	0
Meropenem	12 (100)	0	0
Gentamicin	12 (100)	0	0
Tobramycin	12 (100)	0	0
Ciprofloxacin	10 (83)	2 (17)	0

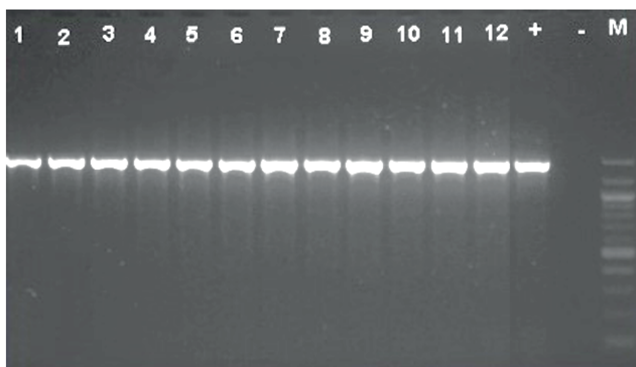

Figure 2. PCR amplification results of 16S rRNA gene from DNA of 12 *P. aeurginosa* isolates. The 16A rRNA gene with a length of 1492 bp. Line M is a DNA marker 100-1500 bp. Line 1-12 are DNA sample of 12 *P. aeruginosa* isolates. Line + is a positive control of 16S rRNA gene. Line – is a negative control.

On the other hand, *S. aureus* isolates also were susceptible to cefoxitin, penicillin, gentamicin, erythromycin, trimethoprim/sulfamethoxazole, chloramphenicol, and clindamycin. The highest resistance level was showed by tetracycline (36%). The finding was the same as previous MRSA study at a referral hospital in Jakarta, that tetracycline was the highest resistance among *S. aureus* isolated from patients at a referral hospital in Indonesia. Tetracycline was one of the most prescribed antibiotic in Indonesia and it might contribute to the high level of resistance.(27) Moreover, we also did not observed MRSA since *mecA* gene was negative in all *S. aureus* isolates. Previously, only 0.8% of MRSA isolated from nasal swabs of surgery patients in referral hospital in Indonesia.(16)

Species identification methods of *S. aureus* among conventional microbiology (coagulase and MSA test), nuc detection and MALDI TOF-MS, demonstrated 100% matches with score values of MALDI TOF-MS were 2.00-2.29 and 2.30-3.00. Coagulase test together with mannitol salt agar improve specificity and sensitivity in culture method for *S. aureus* identification, while nuc gene is the gold standard to identify *S. aureus*.(28) However, the combination of culture techniques and nuc detection was not time efficient. Meanwhile MALDI-TOF provides rapid, accurate and economical values in labor and costs involved. Freshly grown bacteria were placed onto plate and mixed with a matrix that crystallize and entrap the sample. Previous study showed that MALDI TOF-MS has both sensitivity and specificity to distinguish between *S. aureus* and *S. anaerobis* with score values >2.(25) However, there were 3 isolates, which could not be determined by MALDI-TOF MS. This is possibly occurred when the species were not included yet in MALDI-TOF MS database. MALDI-TOF MAS has a limitation that only able to identify bacterial isolate if the peptide match with available spectra of in the database, so that the identification of new isolates is challenging.(26)

P. aeruginosa isolates were identified using Mac Conkey and Kligler Iron Agar as culture identification method, 16S DNA sequencing, and MALDI-TOF MS. All of the *P. aeruginosa* isolates that detected by MALDI TOF-MS showed 100% positivity with conventional culture and 16S sequencing results. Sugar fermentation is the general culture method for *P. aeruginosa* identification. This pathogen is a non-glucose and lactose-fermenter which

Table 6. The 16S rRNA sequencing results of 12 *P. aeruginosa* isolates.

Isolate	Identified Species	% Similiarity	% Identified Sequence	The Number of Identified Bases (bp)
1	*Pseudomonas aeurignosa* LAHAAB 22	100	100	711
2	*Pseudomonas aeurignosa* PE10	100	100	912
3	*Pseudomonas aeurignosa* LYT-4	100	100	911
4	*Pseudomonas aeurignosa* PE10	100	100	912
5	*Pseudomonas aeurignosa* LYT-4	100	100	901
6	*Pseudomonas aeurignosa* PE10	100	100	881
7	*Pseudomonas aeurignosa* LYT-4	100	100	881
8	*Pseudomonas aeurignosa* LAHAAB 22	100	100	839
9	*Pseudomonas aeurignosa* LAHAAB 22	100	100	839
10	*Pseudomonas aeurignosa* LAHAAB	100	100	822
11	*Pseudomonas aeurignosa* B1	100	100	666
12	*Pseudomonas aeurignosa* B1	100	100	666

distinguishes from other Gram-negative bacteria. Thus, it appear as colorless colonies in McConkey and Kligler Iron Agar media. 16S rRNA sequencing is an accepted method for the identification and characterization of *P. aeruginosa*.(29) Score values obtained from MALDI TOF-MS for *P. aeruginosa* were identified as highly probable species identification. *Proteus* sp., followed by *P. aeruginosa* and *Enterococcus*, respectively, were dominant species in suppurative otitis media and the identification was employed by MALDI-TOF MS.(7) MALDI TOF-MS demonstrated the ability to detect high-risk *P. aeruginosa* clones accuaretely, quickly, and inexpensively.(30)

We successfully describe the prevalence and antimicrobial susceptibility profile of *P. aeruginosa* and S. *aureus* from patients with tubotympanic CSOM, which remains limited in Indonesia. Our study will give a recommendation for appropriate medication among CSOM patients. However, further study should be conducted with bigger size sample and middle ear fluid should be collected to elucidate the role of *S. aureus* and *P. aaeruginosa* as causative agents of tubotympanic CSOM accurately.

Conclusion

S. aureus and *P. aeruginosa* were found in patients with tubotympanic CSOM and still susceptible to different antibiotic agents. MALDI-TOF MS results showed 100% matches with conventional culture methodologies and molecular (PCR and sequencing) results. Thus, MALDI-TOF MS demonstrated rapid, accurate and robust to detect *S. aureus* and *P. aeruginosa* compared to conventional microbiology and molecular techniques.

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