

# Identification of Antibiotic-Resistant Bacteria in the Primary Health Center in Bandung (Qualitative study in Puskesmas Ibrahim Adjie)

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**Abstract.** The world is currently facing a serious health threat resulting from antimicrobial resistance (AMR). It is estimated that the global mortality related to AMR is roughly 700,000 per year and is expected to rise to 10 million annually by 2050. Healthcare facilities are among the main contributors of antimicrobial resistance. This study aims to identify the existence of antibiotic resistance bacteria in the air environment of the primary health facility (Puskesmas). Ten samples were collected in 4 different places of indoor environment in Puskesmas Ibrahim Adjie, Bandung, West Java. Antibiotic resistance bacteria (ARB) first selected by growing in 5 different selective media. There are 265 colonies which then selected and identified respectively by using Kirby-Bauer Method with Amoxicillin and Microgen Biochemical Identification. Three dominant bacteria *Stenotrophomonas (Xanthomonas) maltophilia*, *Pseudomonas stutzeri* and *Serratia marcescens*, were found. Those bacteria are not the main pathogenic bacteria but recently recognized as opportunistic pathogen combining a propensity for healthcare-associated infection and antimicrobial resistance.

## 1 Introduction

The world is currently facing a serious health threat resulting from increasing antibiotic-resistance bacteria (ARB) infections. The Wellcome Trust [1] estimated that global mortality related to antimicrobial resistance (AMR) is to be near 700,000 per year and is expected to rise to 10 million annually by 2050. It will accompany with economic burden of 100 trillion. AMR started with the unregulated use of antimicrobials in the human health, veterinary medicine, and production sectors. In Indonesia, self-medication with antibiotic or the use of non-prescribed antibiotics is common. A population-based survey in Yogyakarta city showed a potential increase in self-medication with antibiotics for treatment of minor symptoms such as common cold and headache among Indonesians, particularly in urban areas. The 4-week period prevalence of self medication with antibiotics was 7.3%. The most widely used antibiotics is amoxicillin [2]. Beside high rates of self-medication, common and unnecessary prescription of antibiotics by physicians and over-the-counter purchase of antibiotics are common as well.

Misuse and overuse of antibiotics exerts pressure of selection on pathogen populations that encourage the development of resistance and exchange of resistance genes. The loss of effective antibiotic treatment reduce the ability of healthcare professional to fight infectious

diseases and manage their complications. WHOS's Global Antimicrobial Surveillance System (known as GLASS) show occurrence of antibiotic resistance among 500 thousand people with suspected bacterial infections across 22 countries [3]. The most commonly resistant bacteria were *Acinetobacter spp.*, *Escherichia coli*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Salmonella spp.*, *Shigella spp.*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*.

In Indonesia, limited researches have been done for detecting ARB in human. Bacteria such as *Streptococcus pneumoniae*, *E.coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Klebsiella sp.*, *Proteus mirabilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* have been found resistant to various kinds of single antibiotics. Some of them also classified as multidrug resistant bacteria or resistant to more than one kind of antibiotic [4-6]

Based on some studies, the main contributors of antimicrobial resistance dissemination are animal husbandries, wastewater treatment plants and hospitals[7]. Until now, there is no data about antibiotic resistance bacteria presence in environmental media including water, soil and air in Indonesia. Then, it necessary for initiate research about antibiotics resistance bacteria in environment.

Airborne microbials can move directly, from person to person through respiration, ingestion and dermal contact or indirectly, from waste handling and loading of

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sewage, biosolids or animal wastes. This study taking samples from indoor air environment in one health facility such as primary health facility (called Puskesmas) in Bandung, Indonesia. The aim of this qualitative study to identify the existence of antibiotic resistance bacteria in air environment of the primary health facility.

## 2 Materials and Methods

### 2.1 Sampling

Air samples were collected in Puskesmas Ibrahim Adjie in Bandung city, within 3 different days and from four rooms (Table 1), using air sampler attached with five type of selective agar media plates (Table 2). The health centre was located in Batununggal sub-district, known as one of heterogenous community in Bandung. Selective media was used to selectively targeted certain bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, and *Enterococci*.

**Table 1.** Room type

| Room                   | Code |
|------------------------|------|
| Waiting Room 1st floor | WR1  |
| Waiting Room 2nd floor | WR2  |
| Emergency room         | ER   |
| Delivery room          | DR   |

**Table 2.** Selective Medium and Target Bacteria

| Medium                                 | Organism  |
|--|---|
| XLDA (Xylose Lysine Deoxycholate Agar) | <i>Klebsiella</i> ,<br><i>Salmonella</i>                      |
| CETA (Cetrimide Agar)                  | <i>Pseudomonas aeruginosa</i>                                 |
| VJA (Vogel Johnson Agar)               | <i>Staphylococcus aureus</i>                                  |
| VRBA (Violet Red Bile Agar)            | <i>coli-aerogenes bacterial group</i>                         |
| MCA (MacConkey Agar)                   | <i>E.coli</i> , <i>Shigella</i> ( <i>Enterobacteriaceae</i> ) |



**Fig. 1.** Sampling activity (Waiting Room 1<sup>st</sup> floor)

Sampling situation was shown in Figure 1. Five hundred (500) liters of air sample were used for one type of selective media. Then, the plates were incubated in 37 °C incubator for 24 hours. Colonies growth in media then prepared for isolation (Figure 2). Sampling was conducted in January 2019.

### 2.2 Bacteria Isolation

Variation of colonies from selective media plates were isolated using four quadrant streak methods. The isolation was performed until pure colonies were obtained and confirmed by microscopic (Gram staining) and macroscopic analysis. The pure bacteria isolates were kept in Nutrient Agar slants for downstream analysis.

### 2.3 Antibiotic Susceptibility Test

Antibiotic susceptibility test was done using agar diffusion method (Figure 3). Pure isolates were screened for their susceptibility against antibiotic, in this matter, amoxicillin. Test bacteria (pure isolates) were prepared by inoculating in Mueller-Hinton Broth (MHB) and incubated for 24 hours. The bacteria suspension then diluted to reach 0.08 – 0.13 absorbance using UV-Vis spectrophotometer at 625 nm (equal to 0.5 McFarland). Once the absorbance requirement was fulfilled, 100 µL was mixed with Mueller-Hinton Agar (MHA) at 45-50 °C in sterile plate and gently rotated to ensure even dissemination. Once the MHA solidified, paper discs containing amoxicillin 25 µg were put on the surface of the MHA agar plate. Each plate contained three antibiotic disc (triplicates). Then, all plates were incubated in the 37 °C incubator for 24 hours. After incubation, inhibition zones were measured and each bacterium were categorized as susceptible, intermediate, and resistant. Only isolates categorized in resistant, with diameter <18 mm (based on Clinical and Laboratory

Standard Institute-CLSI) were further analysed to determine their species by using biochemistry characterization analysis and molecular analysis.

## 2.4 Bacteria Identification and Confirmation

The isolated bacteria were then identified by using biochemical test and 16S rRNA molecular marker analysis. The first screening biochemistry characteristic screening was done by using Indol, Methyl Red, Voges-Proskauer, Citrate Utilization (IMViC) and TSIA test. The second biochemistry analysis was done using Microgen ID GN-A and GN-B Kit. The biochemistry kit analysis included the analysis of ability to produce lysine decarboxylase, ornithine decarboxylase, H<sub>2</sub>S, urease, ability to hydrolyze ONPG, reduction of nitrate, indole production, acetoin production, citrate utilization, indolepyruvic acid production, proteolytic enzyme production, ability to inhibit conversion of succinic acid to fumaric acid, fermentation of glucose, mannitol, xylose, inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose, alicin, and conversion of arginine.

Further analysis was done using 16S rRNA sequencing with GGATTAGATACCCTGGTA forward primer (705F) and CCGTCAATTCMTTTRAGTTT reverse primer (907R), and also bioinformatics analysis to clearly determine ambiguous result of previous biochemistry characterization. Sequence trimming done with Bioedit [8] and sequences contig assembling done using ClustalX version 2.0 [9]. The contig sequences were compared with 16S rRNA database in Genbank using BLAST (Basic local alignment search tool) provided by the National Center for Biotechnology Information Server [10].

## 3 Results and Discussion

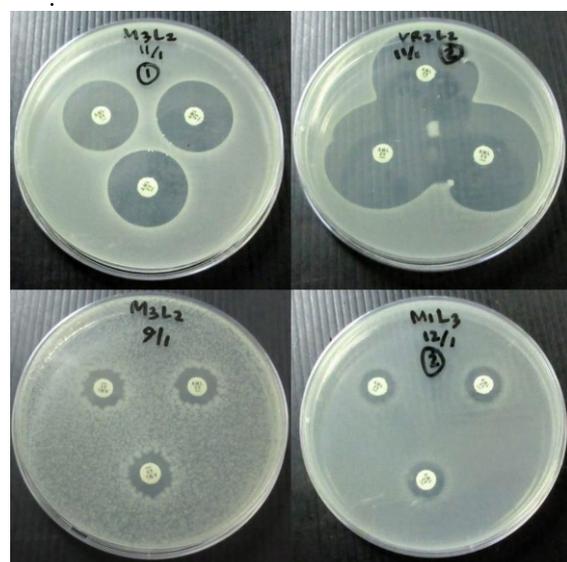
Puskesmas Ibrahim Adjie Bandung (PIA) was chosen because one of the 5 biggest primary health centre, sometimes known as Community Health Centres, in Bandung, West Java with visitor around 100 people per day. It open 6 days a week from 7 am to 4 pm. The PIA building consists of two floors with 11 service clinics. The first floor is occupied by a number of health polyclinics such as General Clinics, Young Toddler Disease Management, Tuberculosis clinic, Laboratory, Emergency Room, Delivery Room, and Pharmacy. On the second floor there is Dental Clinic, Family Planning Service, Underfive Young Toddler (Balita) Service, Mother Child Service, and Counseling room. The PIA also equipped with wastewater treatment plant facilities. Accredited as first level Puskesmas (Utama) with complete facilities as primary health centre. It made the PIA visited by variety age, diseases and community even far away from their service area such as from Bandung Regency. Waiting rooms 1<sup>st</sup> and 2<sup>nd</sup> floor was selected for sampling places because they become gathering place of patients before meeting with the doctor and non patient who accompanied the patient, as well. While

delivery room and 24 hrs emergency room are the place that is expected as main area of pathogen bacteria exposure if compare with other rooms in the health centre.



**Fig. 2.** Medium used for sampling (left) & growth after incubation (right).

Table 3 show amount of colony that can grow in the specific selective medium. All the selective media that is used are the common media for clinical specimens. There are 464 colonies from four different media, with 72,8 % of them grew in Mac Conkey Agar, 20% from Vogel Johnson Agar, 5 % from Violet Red Bile Agar and the rest in Xylose Lysine Deoxycholate Agar. Most of the bacteria colonies that grow have been isolated from waiting room especially from first floor. This room is used as waiting room for almost all activities in the health centre from registration to doctor's, laboratory and pharmacy queue. It also exposed to outdoor environment of the Puskesmas that placed on the side of busy main road.



**Fig. 3.** Antibiotic susceptibility test using agar diffusion method.

From all isolates that test with amoxicillin, 40 pure colonies then identified as gram positive and gram negative which has inhibition zone less than 18 mm and showed amoxicillin resistance. All the bacteria that was found in XLDA medium had grown with inhibition zone 26,5 mm – 34,9 mm and was not categorized as resistant colony.

All presumptive colonies then identified and confirmed with 2 level biochemistry test. We found *Serratia marcescens* and *Stenotrophomonas (Xanthomonas) maltophilia* as confirmed bacteria. Three cultures from three different medium then further identified with 16S rRNA sequencing and bioinformatics analysis which confirmed as *Pseudomonas stutzeri* (*Pseudomonas stutzeri* ATCC 17588 = LMG 11199 16S ribosomal RNA, complete sequence, with 99% query cover and 0 E-value). It was interesting that from selective medium for *Pseudomonas*, Cetrimide Agar, we cannot find any bacteria growth in it. But, from other medium, VJA, VRBA and MCA, the isolates were identified and confirmed as *Pseudomonas stutzeri*.

*Pseudomonas stutzeri* is an anerobic, nonfermenting, a gram-negative and nonfluorescent denitrifying bacterium. It is mostly a saprophyte found in soil and water. *P. stutzeri* is also ubiquitous in hospital environment, so, this species could be considered an opportunistic but rarely leads to serious community or nosocomial acquired infections [11]. Like most *Pseudomonas spp*, it can grow in minimal, chemically defined media with ammonium ions or nitrate and single organic molecule as the sole carbon and energy source. Some *P. stutzeri* strains can grow diazotrophic, this characteristic seems to be rare among the genus *Pseudomonas*. *P. stutzeri* has also been isolated as an opportunistic pathogen from human. Some literatures showed that when *P. stutzeri* is isolated from clinical material it most likely represents colonization in hospitalized patients and uncommonly indicates pathogenicity. During the past decade, there is a significant increase of *P. stutzeri* isolations from clinical material in medical center[11]. The most common reported sites of clinical isolates are surgical wounds, blood, respiratory tract, and urine. Human cases of *P. stutzeri* infection are rare, but some researchs recorded and related it with some cases of infective endocarditis, bone infection, bacteremia, meningitis, pneumonia and induced peritonitis in patients undergoing continuous ambulatory peritoneal dialysis (CAPD) [12-14].

So far, there is no cases reported about *P. stutzeri* infection in human in Indonesia, but it caused an outbreak of freshwater fish in Luwuk Banggai (tilapia and catfish), Bali (tilapia), Jambi (tilapia and catfish) and Tanjung Pinang (catfish)[15].

Summary of sensitivity test for several antibiotics with *P.stutzeri* was written by Lalucat et al[11]. Nearly all studies showed that *P.stutzeri* was sensitive to many more antibiotics than *P.aeruginosa* which is most closely related species and well known as human pathogen. Exclusion, if bacteria isolate were obtained from immunosuppressed patients. Even though, resistant *P. stutzeri* strains have been isolated for almost all antibiotic families, including amoxicillin, with the exception of fluoroquinolones. This suggests that *P. stutzeri* has a wide range of antibiotic resistance mechanisms, at least two mechanisms (i) alterations in outer membrane proteins and lipopolysaccharide profiles and (ii) the presence of  $\beta$ -lactamase that hydrolyze natural and semisynthetic penicillins, broad-spectrum  $\beta$ -lactamase-stable cephalosporins, and monobactams with similar rates.

*Serratia marcescens* is gram-negative bacilli and a member of the genus *Serratia* which is a part of the family Enterobacteriaceae. It occupy various habitats, mainly water, plants, soil, small mammals and hospitalized patients as well. Once considered a harmless saprophyte, *Serratia marcescens* is now recognized as an important opportunistic pathogen combining a trend for healthcare-associated infection and antimicrobial resistance. *S. marcescens* causes both opportunistic and nosocomial infections, and frequent source of outbreak of hospital infection in both adult and paediatric patients. It has been demonstrated to be involved in respiratory and urinary tract infections, bacteremia, septicemia, meningitis, ocular and wound infections, pneumonia, endocarditis, and septic arthritis [16,17]. Infection of *S.marcescens* has been attributed to medical equipment such as nebulisers, bronchoscopes, laryngoscopes, electrocardiogram leads, and contaminated solution including inhalation medications and antiseptics. Environmental sources such as air conditioning units, urine-collecting basins, liquid soap dispensers and even tap water have been implicated as well. Moreover, outbreak among newborn in neonatal intensive care (NICU) and nursery has happened in Saudi Arabia due to *S.marcescens*-contaminated baby shampoo [17].

**Table 3.** Amount of bacterial colony growth in selective medium

| Selective Medium | Total Colony                 |         |         |                              |         |         |                     |         |         |                    |
|------------------|------------------------------|---------|---------|------------------------------|---------|---------|---------------------|---------|---------|--------------------|
|                  | Waiting Room 1st Floor (WR1) |         |         | Waiting Room 2nd Floor (WR2) |         |         | Emergency Room (ER) |         |         | Delivery Room (DR) |
|                  | Batch 1                      | Batch 2 | Batch 3 | Batch 1                      | Batch 2 | Batch 3 | Batch 1             | Batch 2 | Batch 3 | Batch 3            |
| XLDA             | 2                            | 0       | 0       | 1                            | 0       | 0       | 1                   | 0       | 0       | 0                  |
| CETA             | 0                            | 0       | 0       | 0                            | 0       | 0       | 0                   | 0       | 0       | 0                  |
| VJA              | 0                            | 19      | 14      | 3                            | 21      | 34      | 0                   | 3       | 2       | 3                  |
| VRBA             | 5                            | 6       | 1       | 2                            | 5       | 4       | 0                   | 3       | 0       | 0                  |
| MCA              | 23                           | 79      | 46      | 19                           | 73      | 30      | 3                   | 29      | 22      | 14                 |

**Table 4. Antibiotic Consumption per Year in Primary Health Centre of Bandung City\***

| No. | Antibiotic                                | Antibiotic Consumption |         |         |         |
|-----|---|------------------------|---------|---------|---------|
|     |   | 2014                   | 2015    | 2016    | 2017    |
| 1   | <b>Amoxicilin</b>                         |                        |         |         |         |
|     | Amoxicilin capsule 250 mg                 | ND                     | 914000  | 5921300 | 1051900 |
|     | Amoxicilin caplet 500 mg                  | 3514800                | 3076300 | 170600  | 1073900 |
|     | Amoxicilin dry syrup 125 mg/5 ml (bottle) | 85358                  | 68264   | 11710   | 33812   |
|     | Amoxicilin dry syrup 250 mg/5 ml (bottle) | 2700                   | ND      | ND      | 14525   |
| 2   | <b>Chloramphenicol</b>                    |                        |         |         |         |
|     | Chloramphenicol capsule 250 mg            | 98700                  | 85300   | 97900   | 16000   |
|     | Chloramphenicol capsule 500 mg            | ND                     | ND      | ND      | 33900   |
|     | Chloramphenicol syrup 125mg/5ml (bottle)  | ND                     | ND      | ND      | 2110    |
|     | Chloramphenicol ear drops 3% (bottle)     | ND                     | 7656    | 9170    | ND      |
| 3   | <b>Tetracycline capsule 250 mg</b>        | 85200                  | 78600   | 90600   | 57700   |
| 4   | <b>Cefixim tablet 100 mg</b>              | 1470                   | ND      | ND      | 28890   |
| 5   | <b>Erytromycin 250 mg</b>                 | 1100                   | ND      | ND      | 4200    |
| 6   | <b>Ciprofloxacin 500 mg</b>               | 214000                 | ND      | ND      | 122100  |
| 7   | <b>Clindamycin</b>                        |                        |         |         |         |
|     | Clindamycin capsule 150 mg                | 177350                 | ND      | ND      | 55250   |
|     | Clindamycin capsule 300 mg                | 31700                  | ND      | ND      | 81100   |

•Source : Health Profile Report of Bandung City in 2014, 2015, 2016 and 2017

In Indonesia, research about existence *S.marcescens* in hospital patient has been reported. It was found in the specimens of patient treated in both NICU and ICU [19,20]. Important finding from some studies showed the predominant mode of spread of *S.marcescens* is hand-to-hand transmission by hospital or healthcare workers [21]. *S.marcescens* renowned for its ability to exhibit multiple antimicrobial resistance. Studied of old and current strain of *S.marcescens* showed that all strains were resistant to penicillin G, ampicillin, amoxicillin-clavulanic acid, cefazolin, cefamandole, polymixin B, colistin, rifampicin, glycopeptides, fusidic acid, lincosamides, streptogramins, daptomycin, linezolid and cefuroxime[16].

*Stenotrophomonas maltophilia* is a gram-negative bacteria that common found in aqueous habitat, such as water, food and plant rizosphere. This bacteria now become an emerging multidrug resistant global opportunistic pathogen. The increasing incidence of nosocomial and community-acquired *S. maltophilia* infections is of particular concern for immunocompromised individuals [22]. The organism is commonly found in respiratory tract infections, and also cause of bacterimia, urinary tracts infection, meningitis, serious wound infections, mastoiditis, epididymitis, conjunctivitis and endocarditis[23]. Some research show that *S. maltophilia* was resistant multiple antibiotics, including  $\beta$ -lactams, aminoglycosides, carbapenems, tetracycline, quinolone and chloramphenicol.

Instead of finding main pathogenic bacteria with specific medium, this study showed presence of three

opportunistic pathogen bacteria, *Stenotrophomonas (Xanthomonas) maltophilia*, *stutzeri* and *Serratia marcescens*. They recognized recently and numerous studies revealed that they related to nosocomial infection that commonly come from hospital environment, medical equipment and transmission from healthcare workers. Study also showed that there was tendency of increasing antimicrobial resistant of those three. In Indonesia, Gram negative bacteria that cause nosocomial infection tend to resistant to antibiotic that common use for them [24].

Many studies confirming association between increasing use of antibiotics and enhanced antimicrobial resistance. Total consumption of 7 antibiotic in 75-80 primary health centre and government hospital (from 2014-2017) in Bandung can be seen in Table 4. Amoxicillin is a widely-available and used antibiotic in primary health and hospital. Event not really consistent, there was downward trend in Amoxicillin and Tetracyclin use, the most antibiotic first line treatment, especially after year 2016, but not to other kind of antimicrobial. The overall consumption could be double or more, with additional data from private hospital or pharmacies. The potential risk also increase along with high consumption due to self medication practice in Indonesia.

Then, this research become preliminary study to see potential risk contamination of environment with antimicrobials and antimicrobial-resistant microbes, which can accelerate the development and spread of resistance in Indonesia. This is not only threat for human

and animal population but also for natural environment. Research of AMR existence in environmental media such as air and especially water be important to address knowledge gaps and evaluate the potential risk antimicrobial and ARBs in the environment poses to human health and the environmental ecosystem[25].

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