Detection of blaTEM Gene of Klebsiella pneumoniae Isolated from Swab of Food-Producing Animals in East Java

by Eduardus Bimo Aksono
Detection of \textit{bla}_{TEM} \textbf{Gene of \textit{Klebsiella pneumoniae}} Isolated from Swab of Food-Producing Animals in East Java

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(Received 22-01-2018; Reviewed 06-04-2018; Accepted 03-06-2018)

\textbf{ABSTRACT}

\textit{Klebsiella pneumoniae} is one of 9 bacteria resistance to antibiotics in concern. This research aimed to detect any gene of \textit{bla}_{TEM} \textbf{in bacteria of the K. pneumoniae isolated from swab of food-producing animals}. In this study, 195 swab samples were taken from 17 sampling locations. Samples obtained were cultivated on selective medium and had several tests including identification, antibiotic sensitivity testing, Kirby-bauer method against antibiotics of ampicillin, cephalotaxime, amoxicillin, meropenem, and trimethoprim-sulfamethoxazole, and followed by PCR test for detecting the gene that was responsible for the antibiotic resistances. The results showed that 10 out of 195 samples were found to be \textit{K. pneumoniae}, those were 4 samples originated from dairy cows (SP-S1, SP-S3, SP-B2, SP-G4), 2 samples originated from beef (SPT-K1, SPT-K2), 1 sample originated from chickens (A-W5), and 3 samples originated from fish (IN-P2, IN-P3, IN-S3). Most of isolates (9/10) were found to be resistant toward amoxicillin. These isolates were SP-S3, SP-B2, SP-G4, SPT-K1, SPT-K2, A-W5, IN-P2, IN-P3, and the IN-S3 and all of them also showed to be positive of \textit{bla}_{TEM} gene. It could be concluded that most of \textit{K. pneumoniae} isolates from food animals harbour had Extended Spectrum Beta-Lactamase (ESBL) encoding gene.

\textbf{Keywords: antibiotic resistance, ESBL, food-producing animals, Klebsiella pneumoniae, amoxicillin}

\textbf{INTRODUCTION}

\textit{Klebsiella pneumoniae} is a bacterium belonging to the genus of \textit{Klebsiella} a member of the family of Enterobacteriaceae. The bacterium is a normal organism living in tractus digestive so that it can be isolated from animal or human feces (Sussilo et al., 2004). \textit{K. pneumoniae} is a Gram-negative, basil, nonmotile, and one of the important pathogenic bacteria. This species is the agents of various diseases, such as pneumonia, urinary tract infection, bakteremia, infection in wounds, and abscesses of the liver (Karahamathulla et al., 2016). \textit{K. pneumoniae} pose a great impact on the health sector. In a report on global surveillance on antimicrobial resistance carried out by the World Health Organization, \textit{K. pneumoniae} is one of nine bacteria concerned in resistance to antibiotics (WHO, 2014). \textit{K. pneumoniae} was found to be capable of being resistant towards many of the third-generation of cephalosporin antibiotics especially cephalotaxime, ceftazidime, and ceftiraxone (Yeh et al., 2007).

Each bacteria employs different mechanisms in causing resistance to antibiotics. The transfer of antibiotic-resistant genes, especially through plasmids is considered to be one of the important mechanisms in the spreading of antibiotic resistance in bacteria (Apriliani & Pinatih, 2017). This event is known to be mediated by an enzyme of beta-lactamase. The beta-lactamase enzyme is firstly identified in \textit{Escherichia coli}, which is encoded by the \textit{bla}_{TEM} gene. In addition, \textit{bla}_{TEM} gene was also currently found in \textit{K. pneumoniae} (Lazampaia et al., 2014). Treatments of bacterial infections of beta-lactamase producers have so far involved the use of cephalosporins and aztreonam which also belong to the group of beta-lactam antibiotics. In fact, this drug can not kill lactamase producing bacteria as a result of it spread of its resistance spectrum, to penicillins, cephalosporins, and aztreonam so that it called as the Extended Spectrum Beta-Lactamase (ESBL) bacteria. The ability of ESBL strains to hydrolyze beta-lactam antibiotics is generally due to a number of mutations in the gene, and one of which is \textit{bla}_{TEM} gene. These mutations are generally found to be at the active site of the enzyme that leads to a higher enzymatic activity (Yuwono, 2011). The existence of ESBL strain in food-producing animals is reported by Overdevest et al. (2017).

Animals can carry harmful bacteria in their intestines. When antibiotics are given to animals, antibiotics kill most of the bacteria. However, resistant bacteria are survive and multiply. Food-producing animals have been known as reservoirs for ESBL-producing bacteria. Food-producing animals are capable of spreading bacteria that are resistant to antibiotics through feces. Through feces, resistant bacteria contained in...
animal waste and they can migrate around the farms, slaughterhouses or poultry slaughterhouses, and during meat processing. The surroundings of farms and slaughterhouses or chicken slaughterhouses will also be contaminated even though it is far from the source of contamination (Doosti et al., 2014).

Attempts to detect the existence of resistant genes in bacteria from food-producing animals in Indonesia, to our knowledge, remains limited. In fact, the gene detection should allows us to understand the pattern of genes spreading and possibility of the bacteria to gain further antibiotic resistance from certain groups. Accordingly, detection of the bla<sub>TEM</sub> gene group in K. pneumonia strain is important to do.

15 MATERIALS AND METHODS

Sample Collection and Preparation

In this study 195 swab samples were obtained from 17 locations in East Java. Sampling was done by using swab aseptically from dairy cows, beef cattle, broiler, and tilapia in 17 sampling locations. The samples were directly analysed within 30-40 min or then transferred to the lab for further analysis in a ice box.

As many as 195 samples of dairy cows, beef cattle, broilers, and tilapia were cultured by taking 1 ose and then scraped by streak plate technique on selective media of Mac Conkey and EMBA then incubated at 37°C for 24 h. In Mac Conkey media, K. pneumoniae looks pink with colonies culture looks very mucoid and in EMBA media K. pneumoniae looks red to brick (Masruroh et al., 2016).

Characterization of Isolates

Pure bacterial isolates were identified based on morphological characters that included colony morphology, cell morphology, and gram staining tests. Observation of colony morphology was based on the shape, color, and the edge of bacterial colonies. The morphological observations of bacterial cells include the shape and structure of bacterial cells. Furthermore, each isolate was biochemically characterized including carbohydrate fermentation test (glucose, lactose, mannitol, maltose, and sucrose), indole, motility, and citrate (Lestari et al., 2016).

Antibiotic Test

The antibiotic sensitivity test was performed using Kirby-Bauer agar diffusion method (Ningrum et al., 2016). The resulting clear zone was then grouped into sensitive groups (S), intermediates (I) or resistant (R) (Sagita et al., 2015; Kusumaningrum et al., 2016). Selection of antibiotic discs (disks) used previous research and based on some journals for reference. The types of antibiotics used were ampicillin, amoxicillin, cefotaxim, meropenem, and sulamethoxazole-trimethoprim.

Pure cultures were prepared in suspensions with an equivalent of 0.5 McFarland (1 x 10<sup>8</sup> CFU/mL) turbidity. The cultures were taken using sterile swab cotton and distributed by means of a diode on the surface of the Mueller Hinton agar (MHA), and allowed to stand for ≤ 5 min. The antibiotic-containing discs were placed on the top of the MHA, which had been dispensed with pure cultures, at a distance of 25-30 mm. Furthermore, the culture was incubated at 35°C for 24 h (Masruroh et al., 2016).

Genomic DNA Extraction

DNA extraction by adding K. pneumoniae bacteria was performed using QIAamp DNA mini kit 50 (Qiagen, USA) according to manufacturer protocol. Briefly, samples were added with 5 μL lysozyme (5 mg/mL) enzyme and incubated for 30 min at 56°C. Furthermore, the extracted DNA was diluted to 100 μL with a buffer kit. DNA solution used for PCR amplification was as much as 1 μL.

Amplification of blal<sub>TEM</sub> Gene: Polymerase Chain Reaction

For the amplification was performed using Qiagen HotStarTag Master Mix (Qiagen, USA) according to manufacturer protocol with pure genom DNA of K. pneumonia was used as a template. The primers used in this study were shown in Table 1. The amplification steps involved a denaturation process at 95°C for 15 min, 30 cycles denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 2 min followed by the final extension on temperature of 72°C for 10 min (Moestaun et al., 2007).

Amplification product was then separated on 2% gel agarose, stained with gel-red, and visualized using UV light. bla<sub>TEM</sub> gene detection was considered to be positive when bands at 445 bp of apparent size was observed in the gel.

RESULTS

The result showed that 10 out of 195 samples isolated from swab dairy cows, beef cattle, broiler chickens, and fish tilapia were positive K. pneumoniae. The presence of these bacteria on swab samples consisted of dairy cows by 4.8% (4/83), beef cattle by 40% (2/5), chicken broiler by 10% (1/10), and fish by 3% (3/97). All isolates also showed 90% resistance to the amoxicillin.

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primary sequences</th>
<th>Amplicon (bp)</th>
<th>Reference</th>
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<tr>
<td>bla&lt;sub&gt;TEM&lt;/sub&gt;</td>
<td>5'-TCGCCGATACATCTGCTGACGTT-3'</td>
<td>445</td>
<td>Moestaun et al., 2007</td>
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<td>5'-AGGCTCCACGGCTCCAGATTAT-3'</td>
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Table 1. The primers used in this study

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(9/10) and sensitive to other types of antibiotics (Table 2, Figure 1).

Examination of DNA from samples using agarose gel electrophoresis (Figure 2) showed that the bla<sub>RTEM</sub> gene was successfully amplified with bla<sub>RTEM</sub>-F and bla<sub>RTEM</sub>-R primaries. The 10 samples tested by PCR in the study showed a positive result of 9 samples (90%) of the bla<sub>RTEM</sub> gene (Figure 2 and Figure 3).

**DISCUSSION**

The results of biochemical identification showed that *K. pneumoniae* bacteria did not contain indole (−) and Methyl Red (−), contained urea (+), Simmon’s Citrate (+), Voges Proskauer (+), and positive fermentation test of carbohydrate (+). The isolation and identification results were confirmed by *K. pneumoniae* character according to Holt et al. (2000).

*K. pneumoniae* bacteria is a bacterium with a size of 2-3.0 x 0.6 μm and this bacterium is a normal flora in the intestinal and respiratory tracts. *K. pneumoniae* has a large capsule so that in its colonies culture looks very mucoid (Brooks et al., 2005).

Based on the morphology of colonies grown on Mac Conkey agar and biochemical test, *K. pneumoniae* isolates were found from a dairy cow feces, chicken broiler, and tilapia fish as much as 10 out of 45 stool samples. The presence of bacteria of the *K. pneumoniae* on swab samples were 4.8% (4/83) in dairy cows, 40% (2/5) in beef cattle, 10% (1/10) in the broiler chicken, and 3% (3/97) in the tilapia fish.

*K. pneumoniae* bacteria resistance test against antibiotics indicated as much as 90% (9/10) resistant to amoxicillin. The results are similar to the results found by Sagita et al. (2015) that the bacteria *K. pneumoniae* was resistant to the antibiotic amoxicillin. Resistance occurs due to the ability of the bacteria to produce penicillinase enzymes that are capable of breaking down the beta lactam ring. With this effect, penicillin is converted into penicillin acid that is not so active. Resistance is produced by taking action against degraded penicillin by beta-lactamase. Beta lactamase enzymes protect Gram-positive and Gram-negative bacteria. In a Gram-positive bacteria, the enzyme is liberated in the medium and destroys antibiotics before it reached the cell and in gram negative it is located on the route where antibiotics must pass.

**Table 2. Antibiotic inhibition zone interpretation**

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<tr>
<th>Isolate code</th>
<th>SAM (D mm)</th>
<th>AML (D mm)</th>
<th>SXT (D mm)</th>
<th>MEM (D mm)</th>
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Note: R (resistant), I (intermediate), S (sensitive), SP-S (dairy cow in Senduro), SP-B (dairy cow in Batu), SP-G (dairy cow in Grab), SP-T1 (beef cattle), SP-T2 (broiler in Wondo), IN-F (custom tilapia), IN-S (tilapia in Sedili), SAM (ampicillin 10 μg), SXT (sulfamethoxazole-trimethoprim 27.75 μg), CTX (cefoxitin 30 μg), MEM (meropenem 10 μg), AML (amoxicillin 15 μg).

**Figure 1. Inhibition zone diameter interpretation.** 1) SAM (ampicillin 10 μg), 2) SXT (sulfamethoxazole-trimethoprim 27.75 μg), 3) CTX (cefoxitin 30 μg), 4) MEM (meropenem 10 μg), 5) AML (amoxicillin 15 μg).

**Figure 2. Agarose gel electrophoresis 2% product used primer for bla<sub>RTEM</sub> gene detection in Klebsiella pneumoniae bacteria.** Lane M: marker; lane 1-6 sample; lane K (-) negative control. Note: lane 1=SP-S1; lane 2=SP-S2; lane 3=SP-B2; lane 4=SP-G4; lane 5=IN-P2; lane 6=SP-K1.
proceed to reach the target (Sagita et al., 2015). The bacterial isolate that were resistance in this study only occurred against amoxicillin and showed sensitive against ampicillin sulbactam, cefotaxim, meropenem, and sulfamethoxazol trimethoprim. These results are contrary to the study conducted by Sagita et al. (2015) and Ghasemi et al. (2013) stating that the bacteria K. pneumoniae are resistant to cefotaxime antibiotics. Ghasemi et al. (2013) also states that the bacterium K. pneumoniae are 100% resistant to ampicillin antibiotics. Research conducted by Ahmed & Shimamoto (2011) shows that blaTEM genes as antimicrobial resistance found as many as 23 isolates (67.6%) of 34 isolates Gram-negative in case of mastitis in Egypt, and research conducted by Aljanaby & Alhasani (2016) also showed 30 isolates (93.75%) found blaTEM genes from 32 bacterial isolates K. pneumoniae isolated from patients with different clinics infections in Iraq. The majority of ESBL enzymes derived from the TEM type decoded by gene blaTEM, blaTEM gene is a gene causes antibiotic resistance in the plasmids, and it is most often detected in clinical populations of Gram-negative microorganisms (Wilopo et al., 2015).

CONCLUSION

K. pneumoniae can be isolated from swab samples of food-producing animals that is equal to 5.12% (10/195). All of the isolates showed a tendency to be resistant to amoxicillin 90% (9/10). Their resistances also be confirmed by detecting the ESBL-encoding gene ic blaTEM genes. Further research needs to be conducted to study that these bacteria have other ESBL-encoding genes such as CTX and SHV genes. In addition, it is necessary to detect ESBL-producing K. pneumoniae bacteria from the meat of food-producing animals, farm waste, and slaughterhouses, as well as human feces.

CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

ACKNOWLEDGEMENT

Thanks to the Integrated Applied Research Flagship Universities of the Ministry of Research, Technology and Higher Education of the Republic of Indonesia the 2017 Budget that supported this research.

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