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Detection of *bla*$_{TEM}$ Gene of *Klebsiella pneumoniae* Isolated from Swab of Food-Producing Animals in East Java

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ABSTRACT

Klebsiella pneumoniae **is one of 9 bacteria resistance to antibiotics in concern. This research aimed to detect any gene of** *bla*$_{TEM}$ **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 test using Kirby-Bauer method against antibiotics of ampicillin, cefotaxime, amoxicillin, meropenem, and trimetrophrim-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** *K. pneumonia***, 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-KI, SPT-K2, A-W5, IN-P2, IN-P3, and the IN-S3 and all of them also showed to be positive of** *bla*$_{TEM}$ **gene. It could be concluded that most of** *K. pneumoniae* **isolates from food animals harbour had** *Extended Spectrum Beta-Lactamase* (ESBL) **encoding gene.**

Keywords: antibiotic resistance, ESBL, food-producing animals, Klebsiella pneumonie, amoxicillin

INTRODUCTION

Klebsiella pneumoniae is a bacterium belonging to the genus of *Klebsiella* a member of the family of Enterobacteriaceae. The bacterium is a normal organism living in traktus digestivus so that it can be isolated from animal or human feces (Susilo *et al*., 2004). *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 (Rahamathulla *et al*., 2016). *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, *K. pneumoniae* is one of nine bacteria concerned in resistance to antibiotics (WHO, 2014). *K. pneumoniae* was found to be capable of being resistant towards many of the third-generation of cephalosporin antibiotics especially cefotaxime, ceftazidine, and ceftriaxone (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 *Escherichia coli*, which is encoded by the *bla*$_{TEM}$ gene. In addition, *bla*$_{TEM}$ gene was also currently found in *K. pneumoniae*. (Lalzampuia *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 results of it spread of its resistance spectrum, to penicillin, cephalosporins, and aztreonam so that it called as the *Extended Spectrum Beta-Lactamase* (ESBL) bacteria. The ability of ESBL strains to hydrolyze betalactam antibiotics is generally due to a number of mutations in the gene, and one of which is *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 existance of ESBL strain in food-producing animals is reported by Overdevest *et al.* (2011).

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_{TEM} gene group in *K. pneumonia* strain is important to do.

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 tranfered to the lab for further analysis in a cool box.

As many as 195 samples of dairy cows, beef cattle, broilers, and tilapia were cultivated by taking 1 ose and then scrawled by streak plate technique method 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 sulfamethonazole-trimetrophrim.

Pure cultures were prepared in suspensions with an equivalent of 0.5 McFarland (1-2 x 10^8 CFU/mL) turbidity. The cultures were taken using sterile swab cotton and distributed by means of a diole 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 dispersed with pure cultures, at a distance of 25-30 mm. Furthermore, the culture was incubated at 35ºC 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 lyzozyme (5 mg/mL) enzyme and incubated for 30 min at 56ºC. 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 bla_{TEM} gene: Polymerase Chain Reaction

For the amplification was perfomed using Qiagen HotStarTag Master Mix (Qiagen, USA) according to manufacturer protocol with pure genomic 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, extention at 72°C for 2 min followed by the final extension on temperature of 72°C for 10 min (Moenstein *et al*., 2007).

Amplification product was then separated on 2% gel agarose, stained with gel-red, and visualized using UV light. bla_{TEM} 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 1. The primers used in this study

Gene target	Primary sequences	Amplicon (bp)	Reference
bla_{TEM}	F- 5'-TCGCCGCATACACTATTCTCAGAATGA-3'	445	Monstein *et al*., 2007
	R-5'-ACGCTCACCGGCTCCAGATTTAT-3'		

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

Examination of DNA from samples using agarose electrophoresis gel (Figure 2) showed that the bla_{TEM} gene was successfully amplified with bla_{TEM}-F and bla_{TEM}-R primaries. The 10 samples tested by PCR in the study showed a positive result of 9 samples (90%) of the bla_{TEM} 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 bacteria with a size of 2.0-3.0 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 penisilinase enzymes that are capable of breaking down the beta lactam ring. With this effect, penicillin is converted into penicilloid 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

Table 2. Antibiotic inhibition zone interpretation

Isolate code	Antibiotic discs									
	SAM		AML		SXT		MEM		CTX	
	D (mm)	R,I,S	D (mm)	R,I,S	D (mm)	R,I,S	D (mm)	R,I,S	D (mm)	R,I,S
SP-S1	25	S	17	S	28	S	29	S	34	S
SP-S3	24	S	11	R	25	S	30	S	36	S
SP-B2	24	S	13	R	29	S	30	S	35	S
SP-G4	22	S	13	R	23	S	32	S	34	S
SPT-K1	25	S	13.7	R	19	S	34.5	S	40	S
SPT-K2	24.5	S	10	R	20.1	S	30.2	S	30	S
AW-5	22	S	11.2	R	29.7	S	27.7	S	33	S
IN-P2	23	S	12	R	19.9	S	29	S	28	S
IN-P3	21.1	S	10.5	R	22.9	S	27.8	S	29.4	S
IN-S3	24	S	11	R	18	S	34	S	37	S

Note: R (resistant), I (intermediates), S (sensitive), SP-S (dairy cow in Senduro), SP-B (dairy cow in Batu), SP-G (dairy cow in Grati), SPT-K (beef cattle), AW (broiler in Wonokromo), IN-P (customs tilapia), IN-S (tilapia in Sedila), SAM (ampicillin 10μg), SXT (sulfamethonazole-trimetrophrim 27,75 μg), CTX (cefotaxime 30 μg), MEM (meropenem 10 μg), AML (amoxycilin 15 μg).

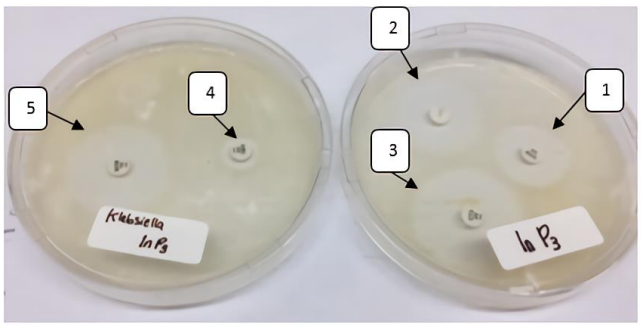

Figure 1. Inhibition zone diameter interpretation. (1) SAM (ampicillin 10μg), (2) SXT (sulfamethonazole-trimetrophrim 27,75 μg), (3) CTX (cefotaxime 30 μg), (4) MEM (meropenem 10 μg), (5) AML (amoxycilin 15 μg).

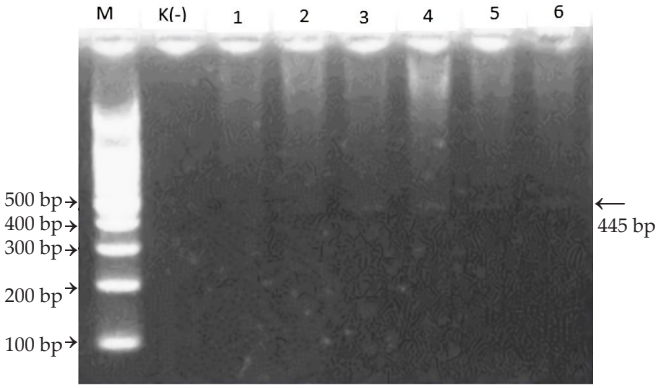

Figure 2. Agarose gel electrophoresis 2% product used primer for bla_{TEM} gene detection in *Klebsiella pneumoniae* bacteria. Lane M: marker; lane 1-6 sample; lane K (-): negative control. Note: lane 1= SP-S1; lane 2= SP-S3; lane 3= SP-B2; lane 4= SP-G4; lane 5= IN-P2; lane 6= SPT-K1.

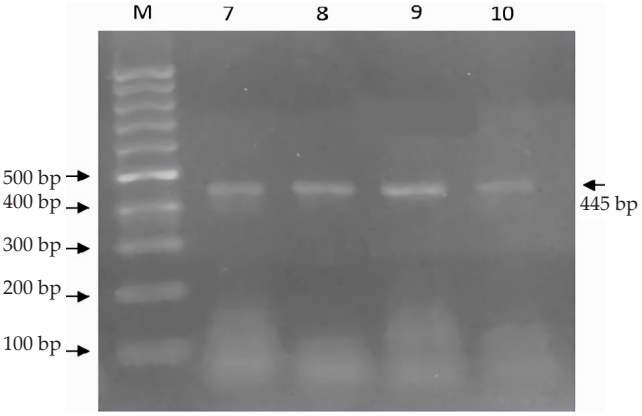

Figure 3. Agarose gel electrophoresis 2% product used primer for bla_{TEM} gene detection in *Klebsiella pneumoniae* bacteria. Lane M: marker; lane 7-10 sample; lane K (-): negative control. Note: lane 7= SPT-K2; lane 8= IN-P3; lane 9=IN-S3; lane 10=AW-5.

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 sulfamethonazole 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 bla_{TEM} 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 bla_{TEM} genes from 32 bacterial isolates *K. pneumoniae* isolated from patients with different clinias infections in Iraq. The majority of ESBL enzymes derived from the TEM type decoded by gene bla_{TEM}. bla_{TEM} 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 ie bla_{TEM} 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 the 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.

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