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ANTIMICROBIAL RESISTANCE DETECTION OF *CITROBACTERFREUNDII* ISOLATED FROM RECTAL SWAB IN EAST JAVA

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ABSTRACT

The aim of this study was to detect *bla*$_{TEM}$gene which the agent of antibiotic resistance in *Citrobacterfreundii* isolated from rectal swab in East Java. A total of 275 rectal swab samples were collected from 12 district of East Java. Then, samples were isolated with selective media and biochemical tests. Antibiotic Resistance test was using the disc diffusion against amoxycillin, amoxycillin-clavulanic acid, ampicillin-sulbactam, cefotaxime, ceftazidime, sulfametaxole-trimethoprim, and tetracycline and then detection of bla $_{TEM}$ gene used PCR. The number of *Citrobacterfreundii* was 7 (2,55 %). Antimicrobial resistance showed 2 broilers and 1 dairy cattle from Surabaya resistant to ampicillin-sulbactam and tetracycline, 4 milkfish from Sidoarjo resistant to amoxicillin and ampicillin-sulbactam. All seven samples were positive *bla*$_{TEM}$ gene with 445 bp size used PCR.

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INTRODUCTION

Citrobacter freundii is a group of Enterobacteriaceae bacteria that can be isolated from stool specimens or intestinal tract of humans and animals (Kao *et al*., 2010). Farm products became the biggest source of fecal contamination that can cause human pathogens. Fecal contamination of water or feces usually presence in the environment or migrate through the water. Still little is known about the effects of antibiotic resistance in animal faeces. Antibiotic resistance in the particles containing the gene resistant genetically and can spread through animal feces in the soil and aquatic ecosystems (Adelowo and Fagade, 2010). Excessive use of antibiotics as well as non-compliance with medication causing downtime residues in poultry meat, eggs and milk or other dairy products (Maynard *et al*., 2003). National Institutes of Health reported that the death rate in the United States reached 90,000 people per year due to antibiotic resistance. Antimicrobial Resistance Bank CDC in 2013 reported That *Citrobacterfreundii* resistant to the antibiotic Amoxicillin, Ampicillin, Cefazolime and Cefoxitin (Guilfoile *et al*., 2007). Antibiotic resistance to beta-lactam antibiotics caused by the production of beta-lactamase, an enzyme that can hydrolyze beta-lactam ring so that the bacterial cell wall synthesis process is not disrupted. One of the enzymes produced by bacteria is the extended-spectrum beta-lactamase, or ESBL which is the result of mutations of beta-lactamase. Most ESBL derived from TEM-type enzyme encoded by the gene *bla*$_{TEM}$. *Bla*$_{TEM}$ genes are genes that cause antibiotic resistance in the plasmid of the most frequently detected in clinical populations of gram-negative microorganisms (Wilopo *et al*., 2015). Based on this background it is necessary to detect the gene encoding bla$_{TEM}$

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as antimicrobial resistance in bacteria *Citrobacterfreundii* isolated from rectal swab of animal that endanger public health.

MATERIALS AND METHODS

Samples

A total of 275 rectal swab samples were collected from 12 district. Rectal swab samples were taken aseptically. The sampling technique is purposive sampling. Rectal swab taken with a sterile cotton swab that has been prepared and then inserted into the sample pot containing physiological saline and has been labelled the previous location. Rectal swabs taken directly from the rectum or spontaneous defecation (rectal toucher). Taken stool samples must be free of urine to prevent bacterial contamination of urine. All samples were stored in the cool box or directly checked exceed 30-40 minutes.

Table 1.Samples

No	District	Sample	Number of sample
1	Surabaya	Catfish, Tilapia fish, Parrot fish, Shark catfish, Shrimp, Dairy cattle, Broiler	33
2	Sidoarjo	Tilapia fish, Milkfish, Catfish, Shark catfish, Beef cattle	31
3	Gresik	Tilapia fish, Shrimp, Milkfish	8
4	Bojonegoro	Tilapia fish, Catfish, Gurame fish	12
5	Blitar	Tilapia fish, Catfish, Gurame fish	2
6	Probolinggo	Tilapia fish, Milkfish, Shrimp, Grouper fish, Mullet fish	11
7	Mojokerto	Tilapia fish, Catfish, Gurame fish, Shark catfish	22
8	Malang	Tilapia fish, Milkfish, Shrimp	8
9	Nongkojajar	Dairy cattle	39
10	Grati	Dairy cattle	48
11	Senduro	Dairy cattle	30
12	Batu	Dairy cattle	31
	Total sample		275

Table 2. Results of resistance phenotype

No	Code	Sample	Location	Resistance phenotype
1	AW-1	Broiler	Wonokromo, Surabaya	SAM, TET
2	AK-5	Broiler	Keputran, Surabaya	SAM, TET
3	SB-3	Dairy cattle	BendulMerisi, Surabaya	SAM, TET
4	SPT.K-1	Beef cattle	Krian, Sidoarjo	AML, SAM
5	IB.S-1	Milkfish	Sidoarjo	AML, SAM
6	IB.S-2	Milkfish	Sidoarjo	AML, SAM
7	IB.S-4	Milkfish	Sidoarjo	AML, SAM

AML= amoxicillin, SAM= ampicillin-sulbactam, TET= Tetracycline

Isolation of *Citrobacterfreundii*

All samples were diluted and then isolated on Eosin Methyline Blue Agar and Mac Conkey's Agar. The inoculated plates were then incubated at 37ºC for 24 hours and then observed macroscopically. Characteristics of bacterial colonies *Citrobacterfreundii* on EMBA appeared diameter of 2-4 mm, smooth, low, convex, moist, metallic green, on MCA appear pink colonies (Holt *et al*., 1994). Furthemore, all bacterial isolated and identified by magnification microscopic examination with negative Gram staining. Negative gram stain showed short stems (bacilli) and then all bacterial isolated and identified by biochemical test (Lay, 1994). Identification test using Triple Sugar Iron Agar, glucose, lactose, sucrose, mannitol, maltose, Simon's Indol Motility, MR- Vogen Prekauer (VP), Simon's Citrate Agar and Urea Agar.

Antibiotic Susceptibility Testing

The antimicrobial sensitivity phenotypes of bacteria lisolates were determined using a Kirby–Bauer disk diffusion assay according to the standards and interpretive criteria described by the Clinical and Laboratory Standards Institute. And used stand art Mac Farland number 0.5 so that the concentration of bacteria to be equivalent to 3.0 x 108 CFU / ml (Mcfarland, 1907). Inspection began with a cotton stick sterile dipped into the bacterial suspension then pressed on the walls of the tube until the cotton is not too wet, then applied to the surface of the Muller Hinton Agar until blended, wait for about 15 minutes, then put the disc containing antibiotics on it and incubated at 37ºC for 19-24 hours. Antibiotic resistance tests against Amoxycillin 20 mg, Amoxicillin-clavulanate 20/10 mg, ampicillin-sulbactam 10µg / 10µg, Cefotaxime 30 g, Ceftazidime 30 g, Sulfametaxole-Trimethoprim 23.75 / 1,25ug, Tetracycline 30 ug. Observations of each antibiotic susceptibility test results based on the diameter of that zone was measured using calipers (Ahmed and Shimamoto, 2011).

Detectionof bla$_{TEM}$ Using PCR

DNA extraction

DNA bacteria extraction were grown on agar overnight at a temperature of 36ºC and then taken colonies using 1µl sterile plastic loop and transferred into 184 mL of sterile distilled water and added to 5 µl lysozyme (5 mg / ml) followed by incubation at 56ºC for 30 minutes.
Furthermore, the DNA was diluted to 100µl with buffer kit (QIAamp DNA mini kit 50). A total of 1µl DNA solution used for PCR amplification (Moenstein*et al*., 2007).

Amplification

Primers used for detection of gene bla $_{TEM}$ shown in Table 3. In the process anneling (attachment) used 1µl DNA solution Hot Star Taq Qiagen Master Mix (Qiagen Nr. 203 445) and 10 pmol primer specific genes reach a final volume of 25µl. Amplification followed denaturation process at 95ºC for 15 min, 30 cycles of denaturation at 94ºC for 30 seconds, anneling at 50ºC for 30 seconds, extension at 72ºC for 2 minutes followed by a final extension at of 72ºC for 10 minutes (Moenstein *et al* ., 2007). The next step was reactingthe PCR products by electrophoresis in 1% (w / v) agarose gel, then stained with ethidium-bromide and visualized using UV light. primersand DNA sequence was shownin Table 2. (Ahmedand Shimamoto, 2011). In genedetectionbla $_{TEM}$ positive results when there are 445 base paresized DNA band (Moenstein*etal*., 2009).

RESULTS

Isolation of *Citrobacterfreundii*

The results of this study from the isolation and identification of bacteria from a total of 275 samples found positive *Citrobacterfreundii* were seven samples (2,55%) that the samples of rectal swab of Boriler from Wonokromo Surabaya (AW-1), Broiler from Keputran Surabaya (AK-5), Dairy Cattle from Bendul Merisi Surabaya (SB-3), Beef cattle from KrianSidoarjo (SPT.K-1), Milkfishes from Sidoarjo (IBS-1), (IBS-2), (IBS-4).

Table 3. Primer used for PCR (Moenstein *et al*., 2007)

Primer	Sekunes (5 to 3)	Gene
TEM-F	TCGCCGCATACACTATTCTCAGAATGA	Bla_{TEM}
TEM-R	ACGCTCACCGGCTCCAGATTTAT	Bla_{TEM}

Antibiotic Susceptibility Testing

Interpretation of the results obtained diameter of antibiotic zone that Broiler from Keputran (AK-5) and Wonokromo (AW-1) and Dairy cattle (SB-3) are resistant to ampicillin-sulbactam and tetracycline, but Beef cattle from Krian (SK-1), Milkfishes from Sidoarjo (IBS-1), (IBS-2), (IBS-4) are resistant to Amoxycillin and Ampicillin-Sulbactam. But on the other antibiotics are still visible intermediete and sensitive.

Detectionof bla_{TEM} Using PCR

PCR result identified of bla_{TEM} in *Citrobacterfreundii*showed on seven samples of broiler from Keputran (AK-5), broilers from Wonokromo (AW-1), Dairy cattle from BendulMerisi (SB-3), Beef cattle from Krian (SK-1), Milkfishes from Sidoarjo (IBS-1), (IBS-2), (IBS-4). The results showed the band in 445 basepare (bp).

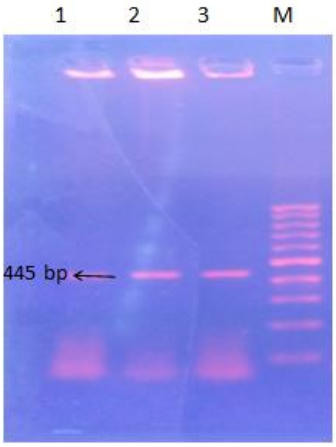

Figure 1. PCR assay to detect *bla* $_{TEM}$ Lane 1= AK-5; Lane 2= AW-1; Lane 3= SB-3; M= Marker

DISCUSSION

The increasing prevalence of ESBL-producing Enterobacteriaceae very harmful to people's health, because the transmission can be derived from food products of animal origin or can be derived from animal faeces. In this study found 7 (2,55%) samples positive *Citrobacterfreundii*from the total 275 samples of rectal swab. Seven samples were broiler from Keputran (AK-5), broiler from Wonokromo (AW-1), Dairy cattle from BendulMerisi (SB-3), Beef cattle from Krian (SPT.K-1), Milkfishes from Sidoarjo (IBS-1), (IBS-2), (IBS-4).

Three samples were AK-5, AW-1, SB-3 found to be resistant to the antibiotic ampicillin-sulbactam, and tetracycline. And SPT.K-1, IBS-1, IBS-2, IBS-4 found to be resistant to the amoxicillin and ampicillin-sulbactam (Table 4). Then followed the PCR test to detect gene bla_{TEM} as agents ESBL and found positive for the band DNA of seven samples in 445 basepare (Figure 1).

In research done by Ahmed and Shimamoto (2011) that the gene coding for antimicrobial resistance bla_{TEM} as found as many as 23 isolates (20.5%) of 34 isolates of gram-negative bacteria in the case of mastitis in Egypt and in *Citrobacterfreundii* found bla_{TEM}genes as much as 3 isolates.Results of research conducted by Kao *et al*. (2010) found a gene *in Citrobacterfreundii* showed bla_{TEM} much as 2 isolates of 30 isolates sampled. *Extended Spectrum Beta Lactamase* (ESBL) derived from TEM-type enzyme encoded by the gene bla_{TEM}. The Bla_{TEM} genes are genes that cause antibiotic resistance in the plasmid of the most frequently detected in clinical populations of gram-negative microorganisms (Wilopo *et al.*, 2015). *Extended Spectrum Beta Lactamase* (ESBL) have the ability to transfer genes from one organism into another organism, so that the spread of resistance very easily occur between strains and even between species (Wilopo *et al.*, 2015). Plasmids are also responsible for coding genes that carry resistance genes to other drug classes (eg, classes (aminoglycoside). This makes selection of antibiotics against ESBL-producing organisms are very limited (Jacob and Munoz, 2005). Members of the family Enterobacteriaceae including *Citrobacterfreundii* often expressing plasmid-encoded βlactamase. Generally ESBL genes derived from TEM-1, TEM-2 or SHV-1 experienced mutase and change the configuration of amino acids around the active site of beta-lactamase. This situation makes the spectrum beta-lactam antibiotics susceptible to hydrolysis by this enzyme (Bush and Jacoby, 2010).

Conclusion

The conclusion of this study shows that seven samples positive *Citrobacterfreundii* found in broiler chickens, dairy cattle, beef cattle and milkfishes in East Java resistance to antibiotics. And *Citrobacterfreundii* showed as ESBL agent to detect bla_{TEM} gene using PCR with 445 basepare size.

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