



The occurrence of multidrug-resistance organisms of ESBL/AmpC-producing *Escherichia coli*, *Klebsiella oxytoca*, and *Kluyvera spp.* isolated from the environment of chicken slaughterhouses in Pondok Rumput, Bogor, Indonesia

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Abstract. *In the last few years, multidrug resistant bacteria increased significantly and contaminated chicken carcass through production chain. This study identified the presence of ESBL/AmpC-producing Enterobacteriaceae collected from the environment of chicken slaughterhouses. Samples were taken before slaughtering process from several points of determined locations. From a total of 84 samples tested, 30 isolates were positive of Enterobacteriaceae comprising of 22 isolates of E. coli, 6 isolates of Klebsiella oxytoca, and 1 isolate of Kluyvera spp. Confirmation test of ESBL/AmpC phenotypic and antibiotic resistant test to 18 kinds of antibiotics were conducted using MASTDISCS D68C commercial kit and Kirby-Bauer disc diffusion susceptibility test with CLSI 2014 interpretation. All of isolates showed resistance to penicillin, ampicillin, amoxicillin, cefotaxime, cefpodoxime, ceftazidime and some isolates showed resistance to streptomycin, gentamicin, trimethoprim-sulphamethoxazol, tetracycline, kanamycin, doxycyclin, colistin sulphate, cephalothin, neomycin, and polymyxin B. This study demonstrated the existence of ESBL/AmpC-producing Enterobacteriaceae that were found on the carcasses container, offal container, floors of carcasses, offal handling area, knives and feathers puller machine. The existence of resistant bacteria in the environment of chicken slaughterhouse can lead to occurrence of antibiotic resistant and transmission of resistance genes to other bacteria that could spread to the carcass that was potentially harmful to humans.*

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INTRODUCTION

Multidrug resistance (MDR) bacteria found clinically are ESBL-producing Enterobacteriaceae, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant Enterococci (VRE). The third group of resistant bacteria is not only present in the hospital environment but also on a poultry farm, chicken carcasses, and other dairy products. Therefore, the spread of MDR bacteria outside the hospital environment has been a serious problem for more than five years. The most likely transmission route for MDR bacteria is from animals to humans through food, especially chicken carcasses and other poultry products

(Gregova *et al.* 2012). ESBL-producing Enterobacteriaceae bacteria that commonly spread at the farm level will be carried and spread on the level of the slaughterhouse. Several studies have examined the existence of Enterobacteriaceae producing ESBL/AmpC on chicken carcasses, but there is still a lack of research data that detects the presence of resistant bacteria in the environment of chicken slaughterhouses that may allow cross-contamination to chicken carcasses.

ESBL genes located on a plasmid can be easily transferred between and among species of bacteria (Overdevest *et al.* 2011). The main resistance mechanisms that need concern are the production of extended-spectrum beta-lactamase (ESBL) enzyme and plasmid-mediated AmpC beta-lactamase group Enterobacteriaceae bacteria. This enzyme has the ability to hydrolyze and inactivate beta-lactam antibiotics, and further resistance is associated with resistance to other antibiotic groups such as fluoroquinolones, aminoglycosides, and trimethoprim/sulfamethoxazole. Previous research has reported the presence of ESBL-producing *E. coli* bacteria in animals throughout the world (Costa *et al.* 2009; Egervarn *et al.* 2014) and in chicken feces taken from slaughterhouses (Masrurroh 2016). Therefore, the purpose of this study was to detect the presence of ESBL/AmpC-producing Enterobacteriaceae (*E. coli*, *Klebsiella oxytoca*, *Kluyvera spp.*) from environmental samples taken at the central slaughterhouse before the slaughtering process in Pondok Rumpit, Kebun Pedes, Bogor, Indonesia and their sensitivity patterns of antibiotic resistance.

METHOD

Sampling was conducted in four chicken slaughterhouses of Pondok Rumpit, Kebon Pedes, Bogor, Indonesia, which produced above 1.000 heads of chickens per day. Samples were taken before the slaughtering process begins. The samples included swab from the floor in carcass and offal handling area, a container of carcasses, containers of offals, knives, a feathers puller machine, and samples from water tank and tap water. The total number of samples was 84 samples. Samples were put in sterile plastic bags and transported to the laboratory using a cooling box. Samples were diluted in 10 mL of 0,1% buffered peptone water. Rinsates (10 mL) were enriched for 24 h at 37°C supplemented with 20 µL cefotaxime (1 µg/mL).

Thereafter the enrichment was streaked onto MacConkey agar (MERCK 1.05465.0500, Germany) containing 1 mg/L cefotaxime and incubated at 37°C for 24 h under aerobic conditions. The colonies which were presumed as *E. coli* will appear as red colonies in the media and are surrounded by a turbid zone. Further works were continued by KOH test, Gram staining, oxidase test (OXOID MB0266A, England), sulfide, indole, and motility (SIM) test, and biochemical test [indole, methyl red, Voges-Proskauer, and citrate (IMViC)]. The colonies that were presumed as *E. coli* were selected and subcultured onto tryptic soy broth (MERCK 1.05458.0500, Germany) at 37°C for 24 h.

The identification of the *E. coli*-like colonies was then confirmed using API 20E (BIOMERIEUX, United States). Isolates were stored in tryptic soy broth containing 20% glycerol at minus 20°C until further workup. Isolation and identification of ESBL-producing *E. Coli* were done by referring to Sudarwanto *et al.* (2016). The isolates were identified for ESBL/AmpC production by using commercial kit MASTDISCS™ ID D68C AmpC and ESBL Detection Discs in accordance with the existing manual and then analyzed using the ESBL computer spreadsheet. The inhibition zones were determined for each isolate using antibiotic discs containing 30 µg of cefotaxime, ceftazidime, or cefpodoxime, either alone or in combination with 10 µg of clavulanic acid (MAST Group, Germany).

Susceptibility to 18 Antibiotics Testing

All isolates which produced ESBL were subjected to susceptibility testing against 18 antimicrobial agents. The concentration of the discs (Thermo Fisher Oxoid, Basingstoke, UK) and abbreviation of antimicrobial agents which were used throughout this paper are: ceftazidime (CTX: 30 µg),

cefpodoxime (CPD: 10 µg), cefotaxime (CTX: 30 µg), cefalotin (KF: 30 µg), penicillin (P: 10 unit), ampicillin (AMP: 10µg), amoxicillin (AML: 25 µg), tetracycline (TE: 30 µg), trimethoprim-sulphamethoxazole (SXT: 25 µg), streptomycin (S: 10 µg), doxycycline (DO: 30 µg), ciprofloxacin (CIP: 5 µg), norfloxacin (NOR: 10 µg), polymyxin B (PB: 300 unit), colistin sulfate (CT: 10 µg), gentamycin (CN: 10 µg), neomycin (N: 30 µg), kanamycin (K: 30 µg) were determined by Kirby-Bauer discs diffusion method recommended by the Clinical and Laboratory Standard Institute (CLSI 2014). As a control, the examination used *E. coli* ATCC 25922 for negative control and *K. pneumonia* ATCC 700603 for positive control. Isolates that showed at least three of the tested antimicrobial grouped into multi-resistant strains.

Data Analysis

Data collected from isolation and identification, phenotyping assay, and antibiotics resistance test were descriptively analyzed to describe ESBL-producing *E. coli* occurrence and its antibiotics resistance profile in environment samples of chicken slaughterhouse in Pondok Rumpu, Kebon Pedes, Bogor, Indonesia.

RESULT AND DISCUSSION

Result

A total of environment sampling (n = 84) that meets the criteria for suspected bacterial Enterobacteriaceae is 30 isolates (35,71%). In detail, the distribution of ESBL bacteria consists of 20 isolates of ESBL-producing *E. coli* bacteria, 3 isolates of ESBL-producing *Klebsiella oxytoca*, and 1 isolate ESBL-producing *Kluyvera spp.* While as many as 4 isolate AmpC-producing *E. coli* and 2 isolates AmpC-producing of *Klebsiella oxytoca* (Table 1).

Table 1 Distribution of ESBL/AmpC in isolates of Enterobacteriaceae

Bacteria	ESBL/ AmpC		Total
	ESBL	AmpC	
<i>E. coli</i>	20 (67%)	4 (13,3%)	24 (80%)
<i>Klebsiella oxytoca</i>	3 (10%)	2 (6,7%)	5 (16,66%)
<i>Kluyvera spp.</i>	1 (3.3%)	0	1 (1,19%)
Total	24 (80%)	6 (20%)	30 (100%)

Table 2 Distribution of ESBL/AmpC bacteria based on sampling location (%)

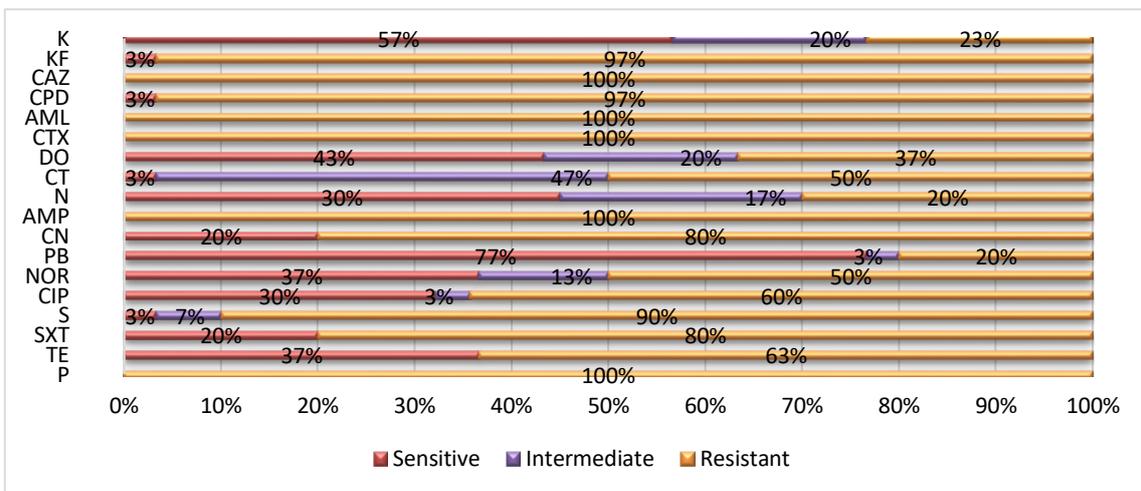
Bacteria	Types of Samples						
	Floor	Carcasses Containers	Offal Containers	Knife	Feather Puller Machine	Water Tank	Tap Water
<i>E. coli</i>							
ESBL	17%	13%	3,3%	10%	17%	3,3%	0
AmpC	7%	0	7%	0	3,3%	0	0
<i>K. oxytoca</i>							
ESBL	3,3%	3,3%	0	0	0	0	3,3%
AmpC	0	0	0	0	3,3%	3,3%	0
<i>Kluyvera spp.</i>							
ESBL	0	0	3,3%	0	0	0	0
AmpC	0	0	0	0	0	0	0

The distribution of ESBL-producing bacteria (*E. coli*, *Klebsiella oxytoca*, *Kluyvera spp.*) was shown in Table 2, which were found on the floor of the handling of carcasses and offal (20%), on the container carcass (17%) and on a container of offals (7%), on the knives (10%), on feathers puller machine (17%), and tank water (3,3%) and tap water (3,3%). The existence of AmpC-producing bacteria (*E. coli* and *Klebsiella oxytoca*) were also found in this study which on the floor of the slaughterhouse at (7%), the container of offals (7%) while the feathers puller machine (7%), and water tank (3,3%).

Discussion

Data showed that *E. coli* and *Klebsiella oxytoca* are bacteria that frequently occur from environment samples Pondok Rumput, Kebon Pedes, Bogor. The highest proportion of ESBL bacteria found in environment samples were 6/30 (20%) on the floor of the handling of carcasses and offal, 5/30 (17%) on the container carcass, and 5/30 (17%) on the feathers puller machine. With the occurrence of resistant bacteria highest in three places, presumably because of frequent contact with internal organs, particularly chicken feces and lack of cleanliness in the area, that would be a high-risk factor for contamination on the carcass.

The use of antibiotics will increase the resistance of some bacteria that can be spread to humans via the environment (air, equipment, water, and soil), work equipment around the production of foods of animal origin, and also the consumption of poultry meat (Levy *et al.* 1976; Geser *et al.* 2012). In most surveillance, there are mentioned pathogenic bacteria and antimicrobial resistance. However, data on the number and mode of transmission by non-pathogenic bacteria carrying antimicrobial resistance determinants are still very rare, such as *Kluyvera spp.* Bacteria of *Kluyvera spp.* found in this research showed multidrug-resistant to 12 kinds of antibiotics. It is quite an alarming finding because of the potential to bring and spread resistant genes to pathogenic and non-pathogenic bacteria around its environment.



Note: AmpC producing Enterobacteriaceae profile against 18 types of Antibiotics: P: penicillin (100%); TE: tetracycline (63%); SXT: sulfametoksasol/trimethoprim (80%); S: streptomycin (90%); CIP: Ciprofloxacin (60%); NOR: norfloxacin (50%); PB: polymyxins B (20%); CN: gentamycin (80%); AMP: ampicillin (100%); N: neomycin (20%); CT: colistin sulphate (50%); DO: doxycycline (37%); CTX: cefotaxime (100%); AML: amoxicillin (100%); CPD: cefpodoxime (97%); CAZ: ceftazidime (100%); KF: cephalotin (97%); K: kanamycin (23%)

Figure 1 Isolates resistance ESBL

Antimicrobial resistance determinants may pose health risks to humans if the bacteria are brought and spread, especially to vulnerable individuals, and lead to the spread through horizontal gene transfer to pathogenic bacteria (Jacoby 2009; Forsberg *et al.* 2012). The finding of the bacteria ESBL/AmpC-producing *K. oxytoca* and *Kluyvera spp.* in the environment also increase the risk of transmission of non-pathogenic

bacteria to pathogenic bacteria. In this study, all isolates of ESBL/AmpC-producing *E. coli* showed resistance to penicillin (100%), amoxicillin (100%), ampicillin (100%), cefotaxime (100%), cefpodoxime (100%), cephalothin (100%), ceftazidime (100%), streptomycin (100%), gentamicin (84,21%), trimethoprim-sulphametoksazol (89,47%). ESBL/AmpC producing *Klebsiella oxytoca* also shows high resistance to the antibiotics penicillin (100%), amoxicillin (100%), ampicillin (100%), cefotaxim, (100%), cefpodoxime (100%), cephalothin (100%), ceftazidime (100%), polymyxins B (66,67%), colistin sulphate (100%), streptomycin (100%), gentamycin (100%), ciprofloxacin (100%), trimethoprim-sulfametoksazol (66,67%). The sensitivity of *Klebsiella oxytoca* is still high on tetracycline (66,67%), doxycyclin (66,67%), and kanamycin (100%) (Figure 1).

Excessive and improper use of antibiotics from a chicken farm level and can then be spreadable to poultry abattoir. Antibiotics are widely available for free use without a prescription, including the use of the most common is a third-generation cephalosporin in combination with other antibiotics. The owner of a poultry farm also frequently uses antibiotics as growth promoters as well as disease prevention by using broad-spectrum antibiotics, for example, tetracycline, ampicillin, gentamicin, ciprofloxacin, and trimethoprim (Been *et al.* 2014). Lack of knowledge regarding good farming practices, education, and free sale of antibiotics are the cause of irrational use. The author also believes that the bad management in poultry abattoir and the waste can be one cause of the spread of these bacteria into the environment. In southeast Asia is still lacking attention to waste compared with western countries, for example, in Bangladesh and India, the slaughterhouse rarely have sewage treatment facilities such as incinerators (Shrivastav *et al.* 2016). Furthermore, the effluent from the slaughterhouse to flow into the river without any specific treatment, where animals around birds, cats, dogs, and the surrounding environment also become agents of the spreader ESBL/AmpC- producing bacteria to other environmental areas.

Wild animals and birds have been reported to be carriers and spreaders of multi-resistant bacteria (Bonnedahl *et al.* 2014). In general, the resistance gene transfer occurs through three mechanisms, namely transformation, conjugation, and transduction. Antibiotic resistance problem mostly comes from horizontal gene transfer between species of bacteria. This mechanism is known to be more efficient for bacteria to adapt to environmental changes than random mutations. Transformation (Marshall *et al.* 2009) and conjugation (Hammerum *et al.* 2014) is the most frequent transfer route. The transfer of antibiotic resistance genes to bacteria pathogenic commensal bacteria depends on the density of the donor and recipient bacteria, the availability of transfer mechanism, nutrition, and selective pressure. The speed in the discovery of new antibiotics is slower than the speed of emergence of resistance, which raises the concern that one day no longer available sensitive antibiotics for infection by resistant bacteria. The whole isolates of Enterobacteriaceae have been detected and showed resistance to the antibiotic cefotaxime, so it is assumed that these bacteria is encoded by CTX-M. This gene is able to hydrolyze cefotaxime compared with the substrate oxymino other β -lactams such as ceftazidime, or cefepime ceftriaxone. Multidrug-resistant pathogens flourish rapidly and become a critical problem in many cases of infectious diseases.

CONCLUSION

The isolates of ESBL/AmpC producing Enterobacteriaceae have shown resistance against all antibiotics. Some of isolates also showed multidrug resistance. It is quite alarming because of the potential to spread resistant genes to pathogenic and non-pathogenic bacteria around its environment.

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