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Detection Of Extended Spectrum *B*-Lactamase (ESBL) Gene Patterns Of *Enterobacteriaceae* In Broiler Chicken Meat Sold In Traditional Markets In The East Surabaya

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ABSTRACT

Chicken meat is one of the livestock commodities that has equivalent nutritional value compared to other meat. Several types of microbes found in food such as *Enterobacteriaceae* (*Escherichia coli*, *Shigella spp*, *Salmonella spp*, *Enterobacter spp*, *Klebsiella spp*, and *Proteus spp*) and other pathogenic microbes easily contaminate chicken meat. Microbial contamination of food comes from the soil, air, water, dust, digestive tract, the touch of human hands, animal beats such as cockroaches and flies, ESBL producing bacteria are one of them. *Enterobacteriaceae* can survive on the surface and water for a long time. In the process of separating viscera from broiler chicken meat, contamination can occur from broiler organ visceral. The three main genes of the ESBL encoder are TEM, SHV, and CTX-M. This study was to analyze the pattern of colonization of ESBL-producing bacteria and ESBL gene patterns in broiler chicken meat. Random sampling was chosen for collecting broiler chicken meat. One hundred portions of broiler chicken meat were taken from the Traditional Market in East Surabaya. This study was an observational analytic study with a cross-sectional approach. Bacteria were growth in TSB media then screened for ESBL production on McConkey Agar with Cefotaxime 2ug/ml. Afterward, continue for phenotypic screening using Double Disk Synergy Test (DDST). Finally, the detection of ESBL gene by using PCR. ESBL-producing bacteria were found in 33 (33%) broiler chicken samples with thirty positive *E. coli*, and three positive samples of *Pseudomonas aeruginosa*. Positive gene detection in SHV genes (1%), TEM genes (18%) and CTX-M genes (24%). ESBL producing bacteria have spread in broiler chicken meat (33%) sold in traditional markets, including *Escherichia coli* and *Pseudomonas aeruginosa*.

Keywords: *Enterobacteriaceae*, ESBL, Broiler Chicken Meat

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INTRODUCTION

Chicken meat is one of the livestock commodities with a nutritional value equivalent to other meat nutritional values (Soegiyono, 2013). The need for *broiler* chicken meat in Indonesia tends to increase by 10% every year. In 2017, the consumption amounting to 5.68 kg per capita/ year increased 573 grams (11.2%) compared to the previous year's expenditure. This increase in demand is in line with Indonesia's growing economic situation. The culinary-based *broiler* meat increased from roadside stalls to shopping centers, which make consumption of chicken meat increased during 2013-2017. *Broiler* meat production in 2017 reached 2.14 million tons, an increase of 97 thousand tons (4.75%) from 2016, which was only 2.04 million tons.

Food consumption of chicken meat containing antibiotic residues will cause health problems. The residual hazards of veterinary drugs can be direct short-term hazards such as allergies, digestive disorders, skin disorders, anaphylaxis and hypersensitivity, and indirect long-term hazards such as microbiological resistance, carcinogenic, mutagenic, teratogenic and reproductive disorders (Ruegg *et al.*, 2013). Various microorganisms from the surrounding environment easily contaminate chicken meat. Some types of microbes found in food are *Enterobacteriaceae* (*Escherichia coli*, *Shigella spp.*, *Salmonella spp.*, *Enterobacter spp.*, *Klebsiella spp.*, and *Proteus spp.*) and other pathogenic microbes. Microbial contamination in food is the result of direct or indirect contamination, with sources of microbial pollution such as soil, air, water, dust, digestive tract, human and animal respiration such as cockroaches and flies (Widyawati P. *et al.*, 2013). Foods derived from animals, especially from poultry, are the main source of infection from the effects of contamination. *Enterobacteriaceae* is a disease-causing agent in foods such as *broiler* chicken meat (Tham *et al.*, 2012; Gines *et al.*, 2015).

Enterobacteriaceae can survive on the surface and water for a long time. Contamination can occur during the process of separating viscera (Koga *et al.*, 2015; Paholewicz *et al.*, 2015). These bacteria can be transmitted from chicken to one another during extraction of feathers, separation of viscera and washing in water (Gregova *et al.*, 2012). *Extended spectrum β -lactamase* and *Enterobacteriaceae* in *broiler* chickens when slaughtered. Most of the isolates identified were *Escherichia coli*, *Enterobacter aerogenes* and *Proteus mirabilis*. In Germany, fresh chicken is the source of the spread of *Enterobacteriaceae* which is carried during the maintenance of feeding and when cutting (Reich *et al.*, 2013).

Extended Spectrum of β -lactamase (ESBL) is an β -lactamase capable of causing bacterial resistance to penicillin; first, second, and third generation cephalosporins; and aztreonam (but not to cefamycin and carbapenem) by hydrolysis of these antibiotics, where enzyme activity can be inhibited by β -lactamase inhibitors such as clavulanic acid (Paterson and Bonomo, 2005).

The three main ESBL coding genes are TEM, SHV, and CTX-M (Johns *et al.*, 2012). These three genes produce ESBL in hydrolyzing β -lactam antibiotics (Sana *et al.*, 2011). The ESBL gene is located in a plasmid, which can be easily disseminated between, and intra bacterial species (Santos *et al.*, 2013). Although the AmpC chromosome gene exists in several *Enterobacter aerogenes* and *E. coli*, the plasmid-bound types can be transferred between bacteria, and it can cause an overall distribution of antimicrobial resistance. Carrier bacteria are not pathogenic, but can cause opportunistic infections in immunosuppressive humans, because *E. coli* is an ESBL producer (Reich *et al.*, 2013).

The phenotypic test for ESBL detection is only used to confirm these bacterial strains including ESBL producers or not. However, it cannot detect the ESBL subtype, which can

only be done by molecular techniques, one of which is the PCR (*Polymerase Chain Reactions*) technique (Sharma *et al.*, 2010).

METHODS

Sampling

This study is an observational analytic-comparative study with cross sectional design. Samples are taken randomly at each traditional market location. A sample of 100 servings of *broiler* chicken is taken from traditional markets in the East Surabaya. Every traditional market, one chance is taken for one sample of *broiler* chicken.

Procedure for Identifying ESBL Producing Bacteria

Broiler chicken samples are carried out according to methods that are routinely carried out by several researchers (Arslan and Eyi, 2011; Nadine *et al.*, 2012; Stuart *et al.*, 2012; Tekiner and Özpınar, 2016) with some modification. *Broiler* chicken meat is cut into small pieces using sterile scissors and placed in a sterile plastic bottle, with a weight of 10 grams of meat. The chicken meat is grinded using sterile stamper then added 20 ml of TSB (*Trypticase Soy Broth*) media, 3 minutes vortex until homogeneous and incubated at 37°C for 4 hours. Afterward, 50 ul was taken and planted scattered to the entire surface of *McConkey* media containing *cefotaxime* 2 ug/ml, incubated at 37°C for 24 hours. The growing colonies thought to be ESBL producing bacteria, were isolated and tested with DDST and bacterial species identification (Nakayama, 2015).

Phenotypic Double Disk Synergy Test (DDST) as Confirmation Test

The bacterial suspension was taken from colonies grown in *McConkey* containing *cefotaxime* 2 µg/ml. Then, inserted into a tube containing 5 ml of *Trypticase Soy Broth* (TSB), turbidity was calibrated with the *McFarland* 0.5 standard. Afterward, it was spread on *Muller Hinton* until evenly distributed and waited for 15 minutes. The antibiotic *Amoxiclav* (AMC) placed right in the middle and the *Ceftazidime*, *Cefotaxime*, *Ceftriaxone*, *Astreonom* placed with a distance of 20 mm (between the center point of the disc to the center of AMC discs). Then, incubated at 37°C for 24 hours. Observation on the widening of the inhibitory zone on CRO, CAZ, CTX and ATZ discs around the edge of the disk facing AMC, against amoxiclav (AMC) disks placed in the center, indicating the production of ESBL enzymes. The widening of the inhibitory zone varies and is called the *key hole effect* (Shaikh *et al.*, 2016).

Biochemical Test of ESBL Producing Bacteria

Biochemical tests were carried out to diagnose the ESBL-producing bacterial species found. The tests were the TSIA Test, Indol, MR (*Methyl Red*) Test, VP Test (*Voges Proskauer*), Citrate Test, and Motility Test. Planting on the media was carried out and identification of the results according to diagnostic guidelines (Tille P.M. *et al.*, 2014).

Genotypic Confirmation (PCR)

Detection of SHV, TEM and CTX-M genes Multiple PCR is by using primers from each of these genes. The PCR process begins with DNA extraction, SHV, TEM and CTX-M gene amplification, and electrophoresis. DNA extraction was carried out according to the procedure described in Bali *et al.* (2010). The extraction results were tested for purity using a spectrophotometer at a wavelength ratio of 260 nm and 280 nm. DNA samples were measured for absorbance. The optical density ratio (OD) 260/280 is calculated, if the result is 1.8-2, the DNA purity level produced is high and can be used further (Herveg and Regaert, 2005; Barbas *et al.*, 2007). PCR of SHV is run with temperature between 60°C-96°C for 10 minutes, TEM between 72°C-96°C for 10 minutes, and CTX-M between 72°C-94°C for 7 minutes. The isolates of ESBL positive *Enterobacteriaceae* bacteria

carrying SHV, TEM and CTX-M genes, from observed DNA bands were included in the tabulation. (Sudarwanto *et al.*, 2015; Lukman *et al.*, 2016).

Data analysis

Data collected from the research results are presented in the form of a table then data analysis is performed.

RESULTS

The study was conducted from March 18 to May 29, 2019 at the Clinical Microbiology Installation of Dr. RSU Soetomo Surabaya and the *Institute of Tropical Disease*, Airlangga University. Of the 100 *broiler* chicken meat samples, 33 (33%) positive samples contained ESBL producing bacteria, which consisted of 30 (30%) samples containing *Escherichia coli* and 3 (3%) samples containing *Pseudomonas aeruginosa*. Analysis of ESBL genes in 33 positive samples shows there is one or more ESBL genes, with the the total number of ESBL genes was 18 TEM, 1 SHV and 24 CTX-M. The presence of a combined gene in each bacterium was found in 15 isolates, which contained a combination of the TEM and CTX-M genes (Table 1).

Table 1.v Distribution of ESBL genes in ESBL producing bacteria contaminated with *broiler* chicken meat sold in traditional markets in the East Surabaya city.

Gene name	Broiler chicken meat (n=100)		Total ESBL	Total gene
	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>		
ESBL Pos	30 (30%)	3 (3%)	33	
ESBL Neg	-	-	67	
Total			100	
SHV	1	-		1
TEM	18	-		18
CTX-M	23	1		24
Total gene	42	1		43

Description: Examination of ESBL genes namely SHV, TEM and CTX-M was carried out by PCR of 33 *broiler* chicken meat.

DISCUSSION

ESBL producing bacteria in *broiler* chickens sold in traditional markets in Surabaya The Eastern region contains 30 % *Escherichia coli* 30 and 3% *Pseudomonas aeruginosa* 3 (Table 1). Research in Netherland on the broad spectrum of beta-lactamase-producing bacteria (ESBL) in animals consumed by humans and contamination of meat can contribute to the increased incidence of infection with ESBL-producing bacteria in humans. Transmission of ESBL genes, plasmids and *Escherichia coli* isolates from poultry to humans, most likely through the food chain transmission of ESBL genes, plasmids and *Escherichia coli* isolates from birds to humans (Leverstein *et al.*, 2011).

Examination of ESBL genes namely SHV, TEM and CTX-M was carried out by PCR. The *broiler* chicken meat containing SHV, TEM, and CTX-M gene are 1, 18, and 24 respectively and there are 15 *broiler* chicken meat containing TEM + CTX-M gene. A research in Germany in 399 chicken meat samples shows as many as 185 confirmed ESBL isolates with 175 (43.9%) samples positive. The majority of isolates were *Escherichia coli*

which produced ESV type SHV 82, CTX-M 77 and TEM 16. There were no observable differences in the prevalence of ESBL between samples of organic and conventional chicken meat. 73.0% (Kola, 2012). Human can be exposed to genes encoding ESBL resistance through food. The SHV variant was first detected in vegetables in Switzerland with blaSHV-12. Similar results were also found in vegetables in Spain (Egea *et al.*, 2011), salad in the Netherlands (Reuland *et al.*, 2014) and South Korea (Kim *et al.*, 2015).

The SHV, TEM and CTX-M genes found in *broiler* chicken meat in the East Surabaya traditional market carried 15 isolates positive of TEM + CTX-M gene. Dagi *et al.* (2015) found that 8% had the CTX-M gene and 77.4% had a combined TEM and CTX-M gene. This is because the plasmid encoding the CTX-M gene is a type of IncFII plasmid, which is a large plasmid that also codes for genes resistant to other groups of antibiotics (Rao, 2012). The CTX-M gene found in other isolates is found in plasmids which have very high transmissible plasmids so that resistance spreads very quickly and efficiently. The bacteria that express CTX-M are mostly co-resistance or multi-resistant bacteria. The TEM and SHV-forming genes are found in the motile genetic element plasmid so that they are easily spread (Livermore and Brown, 2005).

CONCLUSION

There are 33 (33%) of samples of *broiler* chicken sold in traditional markets contain ESBL producing bacteria. ESBL 30 *Escherichia coli* and 3 *Pseudomonas aeruginosa* producing bacteria.. The most genes found are CTX-M, followed by TEM then SHV. The CTX-M gene is suspected of having the ability to spread better than the SHV and TEM genes.

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