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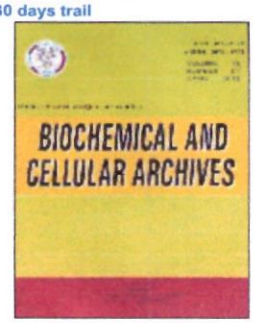

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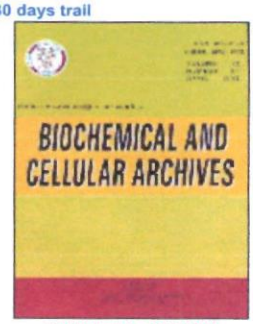

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CASES OF MULTIDRUG RESISTANCE (MDR) AND EXTENDED SPECTRUM BETA-LACTAMASE (ESBL) PRODUCING *ESCHERICHIA COLI* FROM BROILER CHICKEN IN BLITAR, INDONESIA

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ABSTRACT : The study was isolated Escherichia coli from cloacal swab of broiler chicken farms in Blitar area to investigate cases of Multidrug Resistance (MDR) *Escherichia coli* and their *Extended Spectrum Beta-Lactamase* (ESBL) producing *Escherichia coli*. This research was conducted on broiler chicken farms in Blitar district in June until August 2019. Samples using cloaca swabs on broiler chickens were 160 animals from 6 districts in Blitar district. Samples were taken at random and brought to the laboratory for isolation of *Escherichia coli* bacteria. Positive isolates of Escherichia coli were tested for antibiotic sensitivity and Escherichia coli resistant to beta lactam groups were then confirmed using the Double Disc Synergy Test (DDST) confirmation test, to confirm as ESBL-producing bacteria. The results showed the percentage of antibiotic resistance to *Escherichia coli* bacteria in broilers in Blitar District was 88.75% (Ampicillin), 78.75% (Streptomycin), 76.87% (Erithromycin), 50.63% (Tetracyclin) and 75% (sulphamethoxazole-trimethoprim). The incidence of Multi-Drug Resistant (MDR) Escherichia coli bacteria in Broiler chickens in Blitar District was 85.63%, and around 28.75% were *Escherichia coli* bacteria that produce Extended Spectrum Beta-Lactamase (ESBL). In conclusion, the high level of ESBL-producing *Escherichia coli* in broiler chicken cloaca swabs is a threat to public health and the environment and is an important concern to reduce the rate of its spread.

Key words : Broiler chicken, cloacal swab, ESBL, *Escherichia coli*, MDR, public health.

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INTRODUCTION

Antimicrobial resistance (AMR) is the inability of antimicrobials to kill or inhibit bacterial growth so that their use as a therapy for infectious diseases is ineffective. Antimicrobial resistance, especially multidrug resistance is a problem that is difficult to overcome in the treatment of infectious diseases. Multidrug resistance (MDR) organisms are bacteria that are resistant to three or more different antimicrobial classes (Brooks *et al*, 2013). Antibiotic resistance generally occurs due to gene mutations that carry resistance. Mutations in the chromosome system that encode beta-lactamase production by Enterobacter and Citrobacter spp can cause the production of beta-lactamase in very large amounts in a very short time so that it can hydrolyze antimicrobials that are even stable against beta-lactamases such as ceftazidim and cefotaxime (Effendi *et al*, 2018).

Extended Spectrum Beta-Lactamase (ESBL) is an enzyme that can hydrolyze first, second, third, and aztreonam generation cephalosporins (except cephamycin and carbapenem). The presence of ESBL-producing bacteria in an infection can result in treatment failure (CDC, 2003). ESBL-producing bacterial infections have been associated with poor prognosis results. The ESBL enzyme causes some antibiotics to function to treat bacterial infections. Cephalosporin and penicillin antibiotics

are often used to treat bacterial infections, but in the presence of ESBL infections these antibiotics become useless (Kristianingtyas *et al*, 2020).

ESBL-producing bacteria have been detected on farms with increasing incidence in various countries. ESBL-producing bacteria include: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Salmonella enterica*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Enterobacter aerogenes*, *Enterobacter cloacae* (Effendi *et al*, 2018). Test to detect ESBL in Escherichia coli producing ESBL can be performed using the Double Disc Synergy Test (DDST) (Putra *et al*, 2019). The purpose of this study were also to exhibit occurence of multidrug resistant (MDR) of *E. coli* and extended spectrum beta-lactamase (ESBL) producing *E. coli* from broiler poultry farms and understand the hazard for public health problem.

MATERIALS AND METHODS

Isolation and identification of ESBL-producing *Escherichia coli*

This research was conducted on broiler chicken farms in Blitar district in June until August 2019. Samples using cloaca swabs on broiler chickens were 160 animals from 6 subdistricts in Blitar district. Samples were taken randomly, using a sterile cotton swab Amies Viscosa transport media (deltalab, Spain) and stored in a coller box before being taken to the laboratory (Seni *et al*, 2016; Wibisono *et al*, 2020). Cloacal swabs in Amies transport media at cold temperatures were brought to the laboratory for the isolation of Escherichia coli bacteria. Samples of broiler chicken cloaca swabs were cultured on MacConkey selective media. 3 (Oxoid, England) incubated at 35-37^{0}C for 20-24 hours. Pure *Escherichia coli* colonies were identified by Gram staining test (Yanestria *et al*, 2019), then biochemical identification of bacteria was carried out with Indol-Motility, Methyl Red, Voges Proskauer, Citrate (IMVIC) and Triple Sugar Iron Agar (TSIA) tests (Effendi *et al*, 2019; Kristianingtyas *et al*, 2020).

Antibiotic sensitivity test

Positive isolates of Escherichia coli were tested in a positive manner with antibiotic sensitivity testing on Mueller-Hinton agar (Merck MHA Medium, catalog number 1.054.370.500, Germany), as recommended by the Clinical Laboratory Standard Institute (CLSI, 2017) using an available antibiotic disk commercially, the antibiotics ampicillin 10µg (Oxoid, England), streptomycin 10µg (Oxoid, England), erythromycin 15µg (Oxoid, England), tetracyicline 30 µg (Oxoid, England), sulphamethoxazole-trimethoprim 25µg (Oxoid, England). The culture was incubated at 35-37ºC for 18-24 hours. The results of the evaluation after incubation showed that the inhibition zone that appeared in the cup was interpreted based on CLSI guidelines, namely Sensitive, Intermediate, and Resistant.

ESBL-producing Escherichia coli confirmation test

Escherichia coli, which is resistant to beta lactam (ampicillin) group is then confirmed by using a confirmation test, Double Disc Synergy Test (DDST), to confirm it as an ESBL-producing bacteria. This confirmation test is to evaluate the presence of inhibitory zones of ESBL activity with clavulanic acid. DDST confirmation tests used the Amoxycillin-clavulanic 30µg antibiotic disc (Oxoid, England), Cefotaxime 30µg (Oxoid, England), Ceftazidime 30µg (Becton Dickinson, USA), and Aztreonam 30µg (Oxoid, England). The culture was incubated at 35-37ºC for 18-24 hours (Bonnet *et al*, 2000, 2004). The results of the evaluation after incubation showed that the inhibitory zones that appeared in the cup were measured and categorized into three types namely sensitive, intermediate, and resistant based on the guidelines of the Clinical and Laboratory Standards Institute (Wibisono *et al*, 2020).

RESULTS AND DISCUSSION

Isolation results and identification of 160 cloaca swab samples at broiler farms showed 160 (100%) positive isolates of *Escherichia coli* (Table 1). Positive samples of Escherichia coli on MacConkey, as shown in Figs. 1 and 2. In order to be identified with a small round shape and semi-mucoid colony that is reddish pink, it spreads turbidly indicating that *Escherichia coli* bacteria ferments lactose, this is in accordance with Effendi *et al* (2019). The identification of *Escherichia coli* was then confirmed by biochemical tests using IMVIC and TSIA, as shown on Fig. 3. Biochemical tests on the results of this study showed that Escherichia coli was able to produce indole from tryptophan, positive in the Methyl Red test and negative in the Voges-Proskauer test. *Escherichia coli* bacteria also do not use citrate as the only source of carbon. The positive indole test is characterized by the formation of a cerry red ring on the surface of the culture when added with a few drops of Kovac's consisting of p-dimethylaminobenzaldehyde, butanol and acid (Ibrahim *et al*, 2019).

The results of the sensitivity test of *Escherichia coli* bacteria to antibiotics in broiler chickens in Blitar District are shown in Fig. 4. The percentage of antibiotic resistance to *Escherichia coli* bacteria in broilers in Blitar District (Table 2) was 88.75% (Ampicillin), 78.75%

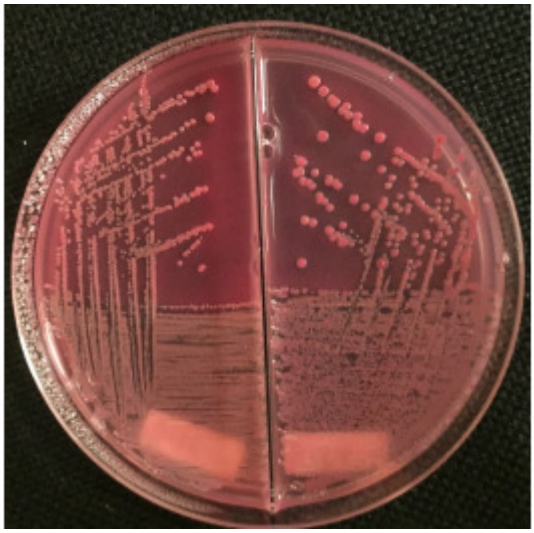

Fig. 1 : *Escherichia coli* on MacConkey Agar.

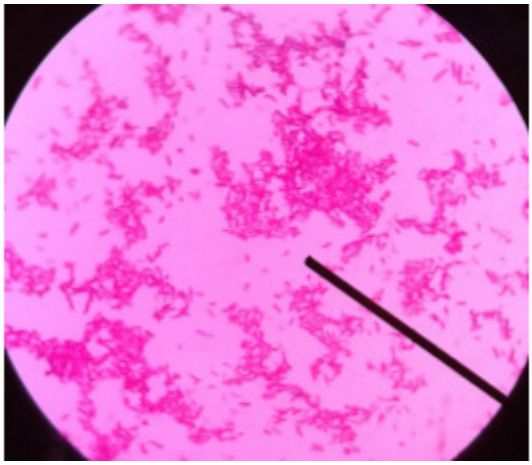

Fig. 2 : Microscopic structure of *Escherichia coli*.

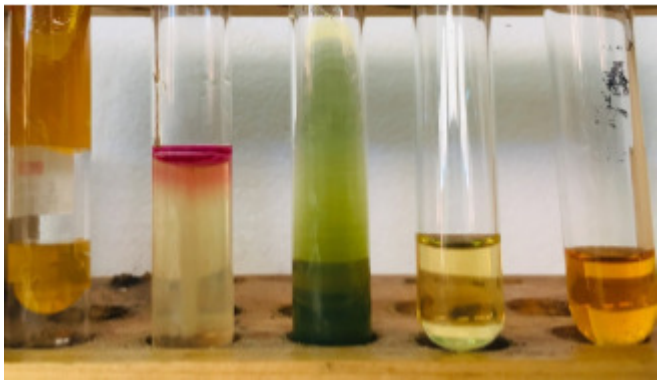

Fig. 3 : Indentification test by IMVIC and TSIA for *Escherichia coli*.

(Streptomycin), 76.87% (Erithromycin), 50.63% (Tetracyclin) and 75% (sulphamethoxazole-trimethoprim). The results of this study are in line with the results of antibiotic resistance to *Escherichia coli* bacteria in broiler chicken meat in the Chicken Slaughter House of Blitar District, namely Erithromycin by 75%, but much higher compared to resistance to ampicillin antibiotics (33.3%), and Streptomycin (50%) (Hartadi, 2019). Other studies stated that the level of resistance of *Escherichia coli* bacteria in Bogor District to nalidixid acid (94.7%), ampicillin (89.5%), enrofloxacin (89.5%), tetracycline (89.5%), erythromycin (86.8%), streptomycin (84.2%), trimethoprim-sulfamethoxazole (76.3%), cephalotin (63.2%), gentamicin (26.3%) and chloramphenicol (21.1%) (Susanto, 2014). High antibiotic resistance in broilers was also reported in Jordan, where the highest resistance levels in sulphamethoxazole-trimethoprim, florfenicol, amoxicillin, doxycycline, and spectinomycin were 95.5%, 93.7%, 93.3%, 92.2% and 92.2% (Canton *et al*, 2012), respectively. Opposite results on the results of research on *Escherichia coli* resistance in broiler chicken faeces showed lower resistance levels also reported in Korea, which showed a resistance level of ampicillin at 50.5% and Tetracyclin at 57.6% (Seo and Lee, 2018).

The results showed that the highest antibiotic resistance of Ampicillin in *Escherichia coli* occurred in Kademangan and Bakung districts which was 100%, the highest Streptomycin resistance in *Escherichia coli* occurred in Kademangan sub-district by 95%, the Erythromycin resistance in *Escherichia coli* was highest in Garum sub-district by the highest 95 percent, the highest Tetracyclin resistance in *Escherichia coli* occurred in Garum sub-district by 75 and Sulphamethoxazole-trimethoprim resistance in *Escherichia coli* the highest was 90% occurred in Kademangan and Bakung sub-districts, as shown on in Fig. 4.

Resistance tests in this study used 5 different types of antibiotics, including beta lactam (Ampicilin), aminoglycoside (Streptomycin) group, macrolide (Erythromicin) group, tetracycline group (Tetracycline), and sulfonamide group (Sulfamethoksazol-Trimetropin), so that the macrolide group (Erythromicin), tetracycline group (Tetracycline) and sulfonamide group (Sulfamethoksazol-Trimetropin), therefore the results of resistance can be seen the existence of multidrug resistance, namely the sensitivity of Escherichia coli bacteria to more than 3 classes of antibiotics. The results showed a high incidence of MDR in broiler chicken farms in Blitar district. The incidence of Multi-Drug Resistant (MDR) *Escherichia coli* bacteria in Broiler chickens in Blitar district is quite high around 85.63%.

The results of this study indicate the incidence of ESBL-producing Escherichia coli in cloaca swabs in broiler chickens with the Double Disc Synergy Test (DDST) method of 46 (28.75%) positive ESBL isolates (Table 1). Cefotaxime synergy with the combination of amoxilin-clavulanate in the form of an expansion of the inhibition zone between the two disks shows that the germ

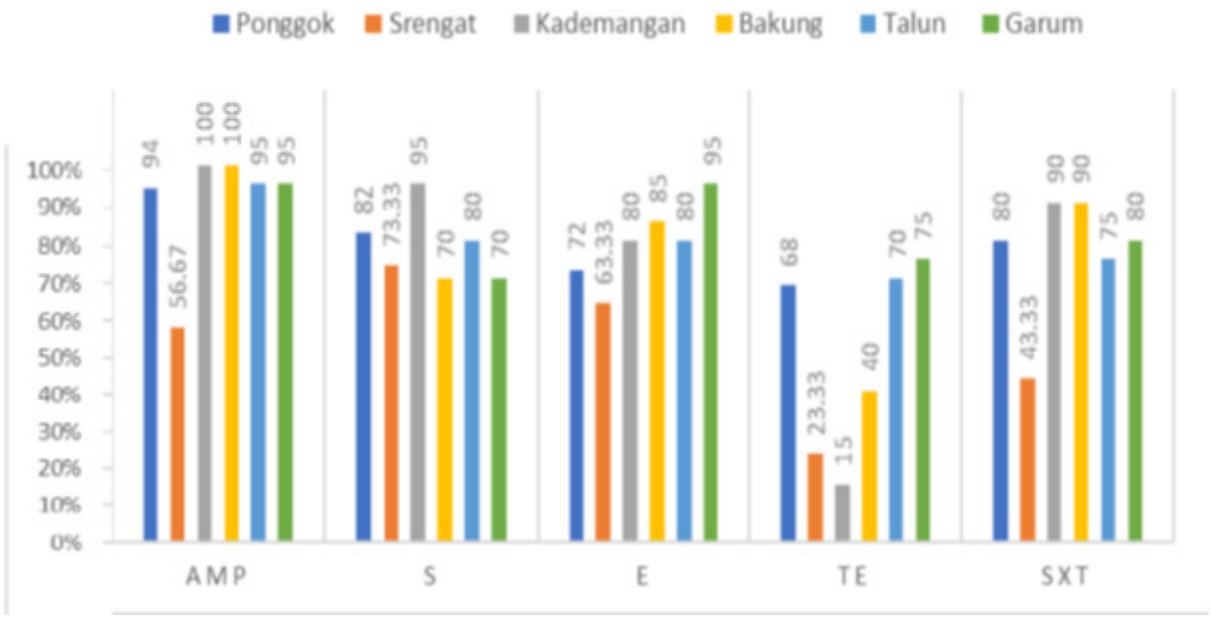

Fig. 4 : Diagram of the percentage of antibiotic resistance in *Escherichia coli* from broiler cloaca swabs on some antibiotics.

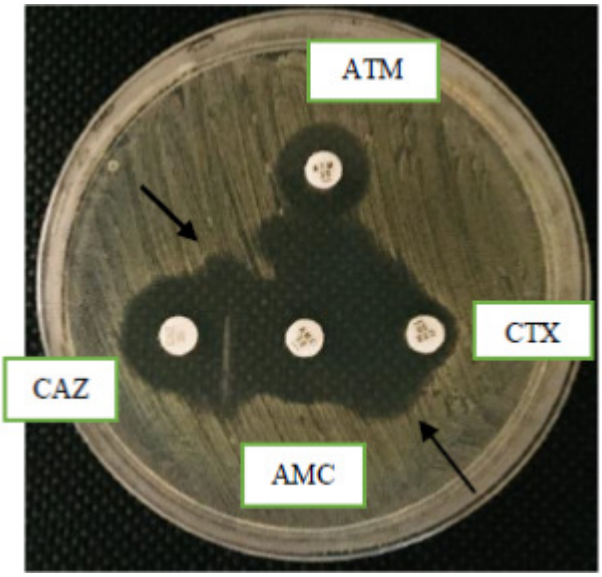

Fig. 5 : ESBL producing *Escherichia coli* by the Double Disc synergy test (DDST) method.

is positive ESBL (Fig. 5), this result is in accordance with the statement of Savira (2014) showed the germ was positive ESBL. Positive results on ESBL-producing bacteria confirmed that there was an increase in the inhibition zone ≥ 5 mm between the diameter of the cephalosporin disk and the cephalosporin-clavulanate combination disk expressed an ESBL positive germ. The incidence of *Escherichia coli* producing ESBL in cloaca swabs in broiler chickens has been reported in Bogor district with an incidence rate of *Escherichia coli* in broiler chicken faeces in Bogor at 6% (Lukman *et al*, 2016), but far smaller than the incidence of Escherichia coli producing ESBL in India on layer chicken about 42% (Brower *et al*, 2017).

In Southeast Asia and Indonesia, based on several reports that E-ESBL has been found, the occurrence is not only in humans but also in animals. Studies on milk samples from dairy farms have reported a positive ESBL of 8.75% (Sudarwanto *et al*, 2015). In cattle faeces samples in slaughterhouses, 15.8% ESBL of E. coli bacteria was found and after 8.6% CTX-M was identified (Sukmawinata, 2015; Sudarwanto *et al*, 2016). The rectal swab molecular identification resulted in 5.21% ESBL E coli positive with 6 isolates of the blaCTX-M gene and 2 isolates of the blaTEM gene (Putra *et al*, 2019). Another study identified ESBL with the Vitek-2 method of rectal swabs of cows resulting in 6% ESBL positive *E. coli* (Putra *et al*, 2020). Animals have the potential as a reservoir for the spread of ESBL E coli bacteria to humans. ESBL *E. coli* can be a threat to human health as well as a dangerous epidemic for the general public. The spread through the food chain associated with ESBL bacteria is a risk that is difficult to handle and control, especially in the era of globalization of trade, hygienic and free bacterial agents in food products of animal origin are important to do so that the spread of ESBL-producing bacteria, from animal to human can be overcome (Widodo *et al*, 2020; Rahmahani *et al*, 2020). ESBL is an enzyme produced in bacterial plasmids that are globally classified into several variants, namely CTX-M, SHV and TEM. CTX-M is an enzyme with an environmental aspect which is currently the most widespread and common type of ESBL associated with the ESBL report (Paterson and Bonomo, 2005). Variants such as CTX-M-15 have been reported to cause outbreaks of infection worldwide, associated with clones causing infection of *E. coli* resistant to antibiotic resistant ST131 antibiotics (Canton

Tabel 1 : Cases of MDR and ESBL producing *Escherichia coli* in Blitar broiler chicken farms.

Location	Sample size	*Escherichia coli* positif	MDR		Confirmed ESBL by DDST	
			Positive	Negative	Positive	Negative
Area Blitar	160	160 (100%)	137(85.63 %)	23(14.37 %)	46(28.75 %)	114(71.25 %)

Table 2 : The sensitivity antibiotics test on Escherichia coli from broiler chicken cloacal swabs in Blitar farms.

Location	Broilers	AMR on Broiler samples									
		AMP		S		E		TE		SXT	
		R	%	R	%	R	%	R	%	R	%
Ponggok	50	47	94	41	82	36	72	34	68	40	80
Srengat	30	17	56,67	22	73,33	19	63,33	7	23,33	13	43,33
Kademangan	20	20	100	19	95	16	80	3	15	18	90
Bakung	20	20	100	14	70	17	85	8	40	18	90
Talun	20	19	95	16	80	16	80	14	70	15	75
Garum	20	19	95	14	70	19	95	15	75	16	80
Total	**160**	**142**	**88.75**	**126**	**78.75**	**123**	**76.86**	**81**	**50.63**	**120**	**75**

Legend : R = resistant, Antibiotic code : AMP = amphicillin, S = Streptomycin, E = erythromycin, TE = tetracycline, SXT = sulphamethoxazole-trimethoprim.

et al, 2012).

The emergence of a plasmid-mediated ESBL variant enzyme such as CTX-M has also been reported since the 1980s (Price *et al*, 2013). Previously found, ESBL type CTX-M has the ability to hydrolyze to cefotaxime. In its development, the type-CTX-M ESBL has the ability to effectively hydrolyze also ceftiofur and cefquinome, broad-spectrum animal cephalosporins, as well as cefotaxime and ceftriaxone. In contrast to the previous ESBL variants, namely TEM and SHV, which only have penicillinase activity. Since around the 2000s, the CTX-M type ESBL has been studied more widely worldwide than the ESBL derivatives of TEM and SHV (Wibisono *et al*, 2020).

The ESBL type CTX-M was originally described as MEN1 (Price *et al*, 2013) and Toho-1 (Barthelemy *et al*, 1992) and was later designated as the CTX-M-1 and CTX-M-44 types. After the emergence of CTX-M-type beta-lactamases which were reported as *Kluyvera* species, members of the *Enterobacteraceae* family intrinsically possessed unique genes on their chromosomes to encode CTX-M-like beta-lactamases such as KLUA-1, KLUA-2, KLUC-1 and KLUG- 1. *Kluyvera georgiana* encoded an enzyme very similar (99%) to CTX-M-8 in the amino acid sequence (Ishii *et al*, 1995), which was first identified in human-isolated Enterobacteriaceae in Brazil (Poirel *et al*, 2002) and later found in poultry and chicken meat samples worldwide (Bonnet *et al*, 2000; Ferreira *et al*, 2014; Wibisono *et al*, 2021). Because the chromosome-like beta-lactamase-mediated CTX-M genes of the *Kluyvera* species have little or no promoter activity at the top of the gene, they tend to be silent. Therefore, *Kluyvera* species are usually susceptible to cefotaxime (Kawamura *et al*, 2014; Decousser *et al*, 2001; Stock, 2005) despite having an intrinsic gene such as blaCTX-M. However, translocation of the chromosome beta-lactamase gene from the *Kluyvera* species to several plasmids with sequence insertion functions, such as ISCR1 (Carter and Evans, 2005) and ISEcp1 (Arduino *et al*, 2002; Saladin *et al*, 2002), which have promoter activity providing resistance to oxymino-cephalosporins through constitutive and multicopy expression of the beta lactamase gene.

Efforts to prevent and control a case of ESBL occurence that results in veterinary public health problem will be very easy if the source of transmission or the origin of the agent is obtained. As with the case of antibiotic resistance, it is very difficult to predict the origin of enzymes, due to differences in amino acids between subgroups and geographical differences from host strains are also very influential (Permatasari *et al*, 2020; Bonnet *et al*, 2004; Quinteros *et al*, 2003). Efforts must be made to prevent and control it by regularly testing the susceptibility to cefotaxime, ceftriaxone or cefepim and ceftazidime regularly, to manage spreading ESBL from for public health purposes (Wibisono *et al*, 2020; Ansharieta *et al*, 2021; Putra *et al*, 2020; Effendi *et al*, 2021).

CONCLUSION

The incidence of Multi-Drug Resistant (MDR) *Escherichia coli* bacteria in Broiler chickens in Blitar district is quite high around 85.63%, while *Escherichia coli* producing Extended Spectrum Beta-Lactamase

(ESBL) in broilers in Blitar District is 28.75% using the ESBL confirmation test method The Double Disc Synergy Test (DDST) of ESBL-producing *Escherichia coli* in this broiler chicken is a threat to public health and the environment, and is an important concern to reduce its spread rate.

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Ethical clearance : Cloacal swabs were used in this study, hence ethical clearance was not necessary. Cloacal swab samples were collected from Blitar area in East Java province, Indonesia.

Conflict of interest : Nil.

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