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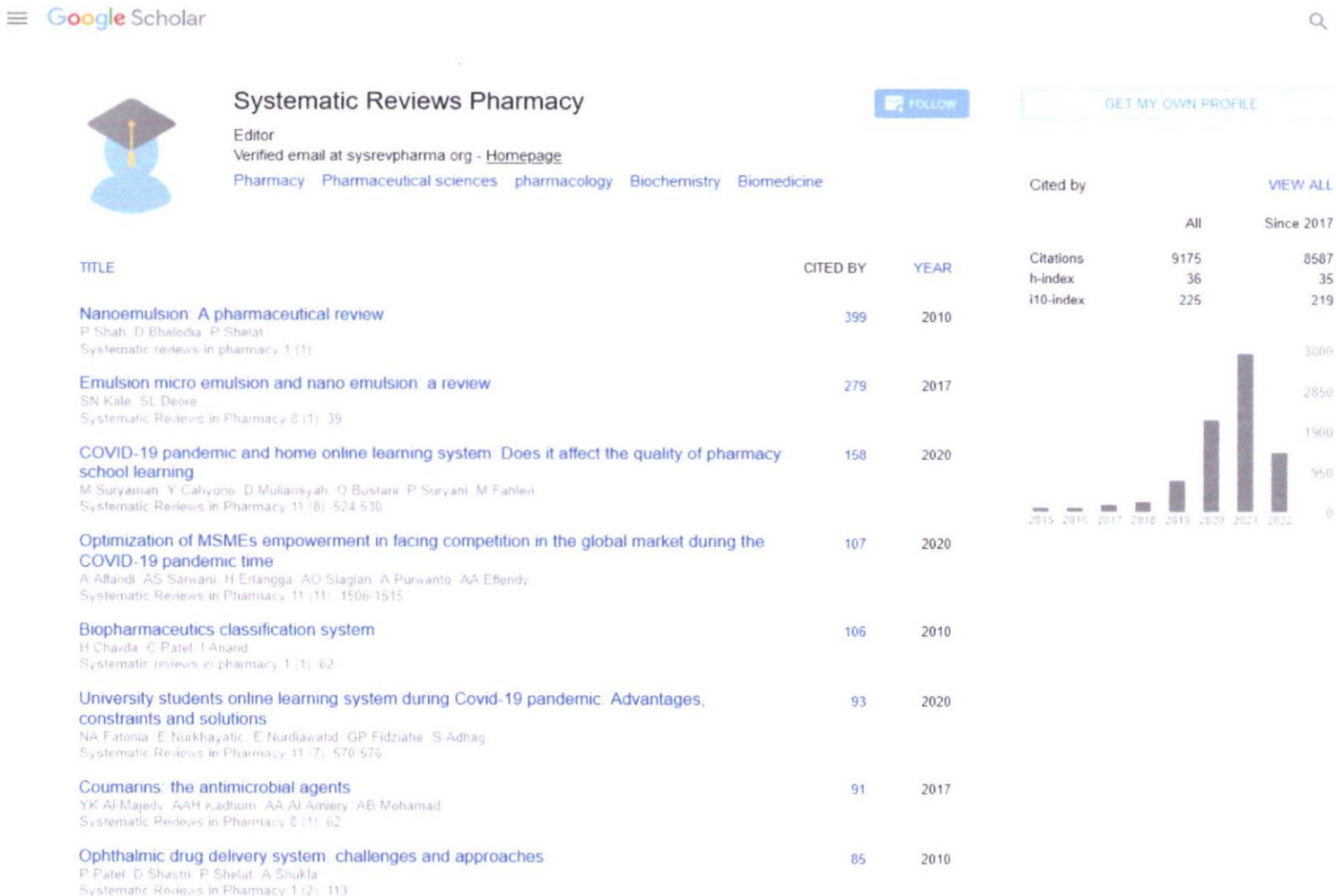


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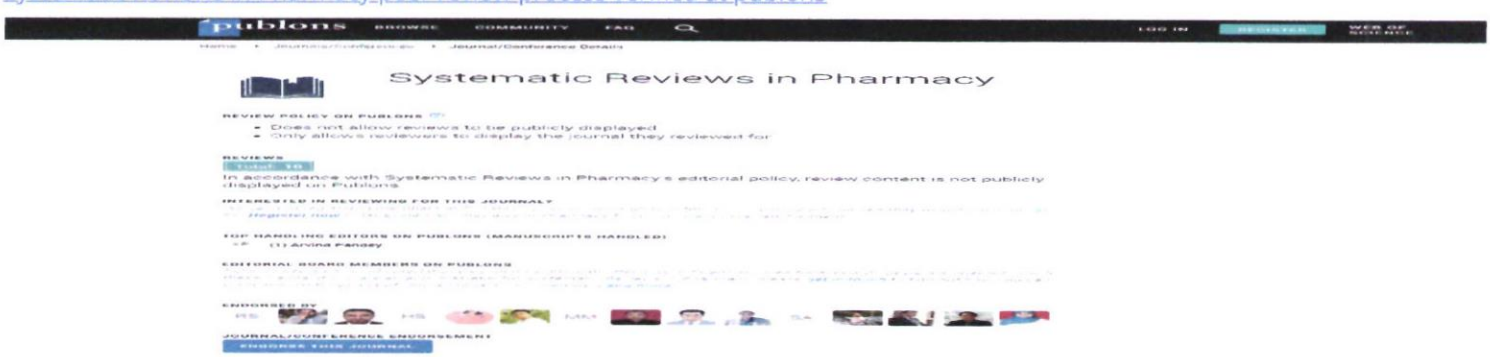


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# CTX Gene of Extended Spectrum Beta-Lactamase (ESBL) Producing Escherichia coli on Broilers in Blitar, Indonesia

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## ABSTRACT

Escherichia coli is one of the causes of infection in humans and animals which can easily cause resistance to antibiotics, one of which is resistance caused by ESBL. This study used 160 broilers cloaca swabs from 6 subdistricts in Blitar District. Isolation of Escherichia coli using Mac Conkey Agar selective media, and identified by biochemical testing of IMVIC and TSIA. Escherichia coli resistance test uses 5 different types of antibiotics, including beta lactam (Ampicillin), aminoglycoside (Streptomycin) group, macrolide group (Erythromicin), tetracycline (Tetracycline) group, and sulfonamide group (Sulfamethoxazole-Trimetropin). ESBL-producing Escherichia coli bacteria were confirmed by the Double Disc Synergy Test (DDST) method. CTX gene characterization using the PCR method. The results showed that 45 isolates (97.8%) were Escherichia coli isolates producing ESBL CTX encoding gene found in broiler cloaca swabs in Blitar district. Positive results on the CTX gene showed electrophoretic results of the samples depicting the same fragment as positive control with a gene length of 550 bp. Escherichia coli which has MDR resistance properties in broiler chickens in Blitar District there were 137 isolates from a total of 160 Escherichia coli. The incidence rate of ESBL-producing Escherichia coli in cloaca swabs in broiler chickens with the Double Disc Synergy Test (DDST) method in the results of this study showed 46 (28.75%) positive isolates of ESBL. These results indicate that the spread of ESBL-producing Escherichia coli is high, therefore it can cause spreading antimicrobial resistance to animal and human health.

**Keywords:** Broiler chicken, CTX gene, ESBL, Escherichia coli, MDR, Human health

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## INTRODUCTION

Extended Spectrum Beta-Lactamase (ESBL) is derived from the mutated beta-lactamase enzyme [1]. Beta-lactamase is an enzyme produced by bacteria that acts to inactivate beta-lactam class of antibiotics [2]. This mutation causes an increase in the enzymatic activity of beta-lactamase so that the enzyme can hydrolyze third generation cephalosporins and aztreonam [1]. ESBL genes are often found, namely the type of cefotaximase (CTX-M), temoneira (TEM), and variable sulfhydryl (SHV). CTX-M type enzymes have hydrophilic ability against cephalosporins, especially cefotaxime so they are called CTX-M [2]. Extended Spectrum Beta-Lactamase is most commonly produced by the Enterobacteriaceae group, especially Escherichia coli and Klebsiella pneumoniae [3,1]. Escherichia coli is one of the causes of infection in humans and animals which can easily cause resistance to antibiotics, one of which is resistance caused by ESBL [1]. ESBL-producing bacteria can also be resistant to the antibiotics class of aminoglycoside, fluoroquinolone, tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim. The multidrug nature of resistance to third generation cephalosporin and other class antibiotics is often found in ESBL-producing bacteria [4].

The presence of ESBL-producing bacteria in an infection can result in treatment failure. Antibiotic resistance causes a decrease in the effectiveness of treatment, increases transmission of infection, increases mortality, and increases the cost of health care, while the discovery of new antibiotics is getting less and less [5,6]. The nature of ESBL-producing Escherichia coli resistance to antibiotics results in limited treatment options [7]. Escherichia coli which produces ESBL in livestock and the environment makes a potential risk factor for public health and is a factor in the transmission of antibiotic resistance in humans [8]. The existence of ESBL-producing Escherichia coli in poultry in Indonesia has been reported in broiler feces in chicken slaughterhouses in Bogor by molecular detection (genotypic) examination using a PCR of 6% [7]. and clinical microbiology (phenotypic) examination by the double disc method of 25% [4].

## MATERIALS AND METHODS

### Isolation And Identification of Escherichia coli

The total sample used in the study was 160 broilers cloaca swabs from 6 districts in Blitar District. Isolation and identification of Escherichia coli was carried out by [9]. Cloacal swab sampling used the Amies Swab Viscosa (deltalab, Spain), and during the trip to the laboratory it was stored at cold temperatures [9,10,11]. Isolation of Escherichia coli bacteria using selective media Mac Conkey Agar no. 3 CM0115 (Oxoid, England) was incubated at 35-37$^{o}$C for 20-24 hours. Pure colonies of Escherichia coli were identified by biochemical testing of IMVIC (Indol-Motility, Methyl Red, Voges Proskauer, Citrate) and TSIA (Triple Sugar Iron Agar)[12].

### Confirmation Test of ESBL-Producing Escherichia coli And Antibiotic Resistance

Resistance tests in this study used 5 different types of antibiotics, including the beta lactam (Ampicilin) group, the aminoglycoside group (Streptomycin), the macrolide group (Erythromicin), the tetracycline group (Tetracycline), and the sulfonamide group (Sulfamethoksazol-Trimetropin) so 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. ESBL-producing Escherichia coli bacteria isolated from broiler chicken cloaca swabs were confirmed by the Double Disc Synergy Test (DDST) method on Mueller-Hinton agar according to recommendations from the Clinical Laboratory Standard Institute (CLSI) [13] using commercially available antibiotic disks, namely Amoxycillin-clavulanic 30µg (Oxoid, England), Cefotaxime 30µg (Oxoid, England), Ceftazidime 30µg (Becton Dickinson, USA), and Aztreonam 30µg (Oxoid, England). This confirmation test with DDST method was conducted to evaluate the presence of inhibitory zones of ESBL activity with clavulanic acid using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Merck, Germany) [12,13]. The results of the evaluation after incubation showed a synergy between Cefotaxime / Ceftazidime with the Amoxycillin-clavulanic combination in the form of an increase in inhibition zone ≥ 5 mm between the diameter of the cephalosporin disk and the cephalosporin-clavulanate combination stated the Escherichia coli positive ESBL bacteria [13,14, 15].

### CTX Gene Characterization Using The PCR Method

The ESBL-producing Escherichia coli bacteria that has been phenotypically confirmed by the DDST method, then genotypically confirmed by further analyzing the presence of ESBL CTX subtypes gene using molecular identification of PCR. Bacterial DNA was isolated with a mini QIAamp® DNA kit (QIAGEN, Germany). Escherichia coli ATCC 35218 was used as a positive control standard for strains of ESBL-producing bacteria and Escherichia coli ATCC 25922 was used as a negative control. The primers used to encode the encoding gene are CTX-MA (CGCTTTGCGATGTGCAG), CTX-MB (ACCGCGATATCGTTGGT), with denaturation temperatures of 94 $^{o}$C, 2 minutes; extended denaturation of 94 $^{o}$C, 1 minute; annealing 52 $^{o}$C, 30 seconds; extension 72 $^{o}$C, 45 seconds; extended extension 72 $^{o}$C, 5 minutes, this reaction is carried out for 30 cycles. PCR results were visualized by electrophoresis using agarose gel 2% (Invitrogen, USA) [16,17].

## RESULTS

Isolation and identification of broiler chicken cloaca swabs from a total of 160 samples showed 100% (Table 1) of the presence of Escherichia coli presumptive isolates on MacConkey Agar media. Escherichia coli colony has a small round shape and semi-mucoid, reddish pink colony. The bacterial culture was then confirmed by biochemical testing using IMVIC and TSIA. The pure colony of Escherichia coli was then tested for antibiotic sensitivity. The incidence of Multidrug Resistant (MDR) Escherichia coli as shown on Figure 1, in Broiler chickens in Blitar District is quite high around 85.63%, among others in Ponggok sub-district by 90% (45/50), in Srengat sub-district by 50% (15 / 30), in Kademangan sub-district by 100% (20/20), in Bakung sub-district by 100% (20/20), in Talun sub-district by 90% (18/20), and in Garum sub-district by 95% (19/29 ), as shown in Table 1. Escherichia coli which have MDR resistance properties in broiler chickens in Blitar District there were 137 isolates from a total of 160 Escherichia coli. The incidence of MDR in Escherichia coli on broiler chickens for each district was shown on Figure 2. The percentage of positive MDR events was more dominant in all districts in Blitar district which were used as samples in this study. Kademangan and Bakung Subdistricts had the highest percentage of MDR Escherichia coli in broiler chickens by 100%, followed by Garum subdistricts (95%), Ponggok and Talun subdistricts (90%), and the lowest events occurred in Srengat subdistrict (54.55%).

The incidence rate of ESBL-producing Escherichia coli in cloaca swabs in broiler chickens with the Double Disc Synergy Test (DDST) method in the results of this study showed a figure of 46 (28.75%) positive ESBL isolates (Table 1). The incidence of Escherichia coli producing ESBL in Broiler chickens in Blitar is quite high, including in Ponggok sub-district by 8% (4/50), in Srengat sub-district by 16.67% (5/30), in Kademangan sub-district by 85% (17/20) ), in Bakung sub-district by 100% (20/20), and in Talun sub-district and Garum sub-district there were no ESCHB-producing Escherichia coli events detected. Cefotaxime synergy with the combination of amoxilin-clavulanate in the form of an expansion of the inhibition zone between the two disks showed that the isolate was positive ESBL (Figure 3 and Figure 4). Almost ESBL producing E. coli detected have CTX gene 97.8% (45/46), as shown on Table 1 and revealed on Figure 5.

## DISCUSSION

The current concern about aspects of human health and animal health is the emergence of strains of Escherichia coli which have the ability to resist some antibiotics, this can occur because of the pattern of antibiotic use on farms continuously both in treatment doses and their use as feed and growth additives. promoter. Excessive use of antibiotics and outside the control limit has the potential to cause antibiotic resistance and is likely to spread the resistant gene to other bacteria, so that it can affect treatment failure in humans and in animals [18,19]. Multiple drug resistance, including ampicillin, streptomycin, and tetracycline derivatives can almost always be found in every case of bacterial resistance to antibiotics, especially for Escherichia coli and Salmonella spp [20,21,22]. This statement supports the test results, because there are isolates that show Escherichia coli resistance patterns. against several antibiotic preparations at once, namely erythromycin, ampicillin, tetracycline, and streptomycin. The occurrence of resistance is not a new phenomenon and is a problem for the whole world [23, 24].

The incidence of Escherichia coli producing ESBL in the cloacal swab in broiler chickens is in accordance with the incidence of Escherichia coli in the faeces of broiler chickens in Bogor by 6% [7], however much smaller than the incidence of Escherichia coli producing ESBL in India in 6 broiler chickens around 87% [20]. In this study also conducted research on ESBL producing E. coli. This was based on research conducted by [25] which stated that all ESBL-producing isolates were also MDR isolates that isolated from layer chicken. The nature of multidrug resistance of these isolates may be explained by the fact that ESBL is mediated by plasmids carrying multiresistant genes by plasmids, transposons and integrons and also they are ready to be transferred to other bacteria, not necessarily same species. Bacteria with various resistance to antibiotics are widely distributed in animals and the

environment. The facts supported by recent surveys from another layer chicken farms [26], have illustrated an alarming trend related to resistance among ESBL-producing organisms isolated from animals and the environment.

Escherichia coli producing ESBL coli in broiler chickens for each subdistrict was shown in Figure 4. The incident occurred in 4 sub-districts of the 6 examined subdistricts, including Ponggok, Srengat, Kademangan, and Bakung. The highest percentage of events occurred in Bakung sub-district (100%), and in the Talun and Garum sub-districts the ESBL-producing Escherichia coli was not found in broiler chickens.

Molecular identification as shown in Table 1 showed that 45 isolates (97.8%) were isolates of Escherichia coli bacteria that were produced by CTX gene encoding ESBL, as shown on Figure 5. The CTX encoding gene is most commonly found in Escherichia coli. CTX type enzymes have a hydrophilic ability against cephalosporins, especially cefotaxime, so called CTX [27]. The molecular identification results shown in Figure 5 showed the visualization of the CTX gene fragment band. Positive results on the CTX gene showed electrophoretic results of the samples depicting the same fragment as positive control with a gene length of 550 bp. The negative results of the CTX-M gene on ESBL showed that the electrophoresis of the sample did not represent the same fragment as positive control [15].

Our findings from molecular identification illustrate that CTX gene was the most common genotype on E coli isolated from broiler chicken, almost 97.8% (45/46), as shown on Table 1 and revealed on Figure 5. This finding was similar to in Turkey [28] and around the world that also report CTX as the dominant ESBL genotype [29,30]. Similarly, in animals, the prevalence of ESBL-producing *E. coli* in China has increased rapidly in years with CTX being the main gene coding that applies to ESBL [31]. It is known that, in general, ESBL genes are located on plasmids which can spread easily between commensal and pathogenic bacteria in poultry farms and the environment.

Our results were similar to previous studies in the United Kingdom showing that the blaCTX-M-1 gene was detected in 5 genes from 32 (15.6%) samples of broiler manure and showed this the dominant order type in the UK broiler the faeces is CTX-M-1 [32]. Another study conducted in Korea reported that E. coli from 9 (9.0%) of 100 chicken feces samples examined resulted in ESBL-type CTXM, namely CTX-M-14 (n = 4), CTX-M-15 (n = 4), and CTX-M-1 (n = 1) [33]. The rapid increase and spread of CTX-M worldwide in E. coli is a matter of concern in both human and veterinary medicine. For this reason, E. coli producing CTX-M has been reported which can transfer this determinant to others such as commensal Enterobacteriaceae, such as K. pneumoniae, or to pathogens such as Shigella or Salmonella spp. [34].

The results showed that 137 isolates from a total of 160 E coli isolates described an MDR pattern with at least three antibiotics. ESBL production is often mediated by plasmids that often carry genes encoding resistance to other drug classes, such as fluoroquinolones, aminoglycosides, sulfa derivatives and trimethoprim [35]. Drugs of this class are often used in broiler production, co-selection through the use of these drugs may play a role in the selection of ESBL-producing isolates [36]. The presence of the CTX gene for ESBL-producing E. coli in broilers in Blitar can be an important risk factor for environmental pollution and spread to the food chain. ESBL contamination generated by E. coli in poultry products can easily be acquired from poultry to their farm environment through contact with contaminated water or during house cleaning [37]. Surface water contaminated with faecal bacteria has long been a problem because water quality has the potential for disease transmission [38].

Human populations can be exposed to antimicrobial resistant bacteria through contaminated surface water as it is an important source of drinking water production and is used for recreational activities and crop irrigation. In addition, animals can also be exposed to these bacteria by drinking from or foraging in contaminated water [39]. ESBL producers are expected to increase in the future in animals and humans [7, 33, 40]. However, more research is needed to understand how persistence and spread can be minimized.

## CONCLUSION

Molecular identification of CTX gene in ESBL-producing Escherichia coli on broilers cloacal swabs in Blitar district, as many as 45 (97.8%) out of a total of 46 ESBL-producing Escherichia coli isolates found in broiler chicken cloacal swabs in Blitar district. MDR distribution was found in 6 sub-districts in Blitar district from 6 sub-districts used as sampling areas, while the distribution of ESBL-producing Escherichia coli events was found in 4 sub-districts in Blitar, namely Ponggok, Srengat, Kademangan and Bakung. These results indicate that the spread of ESBL-producing Escherichia coli was sufficiently large, therefore that it can cause high antimicrobial resistance to animal and human health. This spread ESBL enzymes among isolates that produced by healthy broilers can be a problem for food safety, the environment, humans, animals, and other pathogenic bacteria.

## DECLARATION OF CONFLICT OF INTEREST

None.

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**Table 1**. Data of ESBL producing *Escherichia coli* and MDR on Broiler chickens in Blitar

| Location | Sample size | Positive of *Escherichia coli* | MDR | | ESBL | | CTX gene |
|---|---|---|---|---|---|---|---|
| | | | Positive | Negative | Positive | Negative | |
| Ponggok | 50 | 50 | 45 (90 %) | 5 (10 %) | 4 (8 %) | 46 (92 %) | 4 (100 %) |
| Srengat | 30 | 30 | 15 (50 %) | 15 (50 %) | 5 (16.67 %) | 25 (83.33 %) | 5 (100 %) |
| Kademangan | 20 | 20 | 20 (100 %) | - | 17 (85 %) | 3 (15 %) | 16 (94.1 %) |
| Bakung | 20 | 20 | 20 (100 %) | - | 20 (100 %) | - | 20 (100 %) |
| Talun | 20 | 20 | 18 (90 %) | 2 (10 %) | - | 20 (100 %) | - |
| Garum | 20 | 20 | 19 (95 %) | 1 (5 %) | - | 20 (100 %) | - |
| **Total Broiler** | **160** | **160 (100%)** | **137 85.63 %** | **23 14.37 %** | **46 28.75 %** | **114 71.25 %** | **45 97.8 %** |

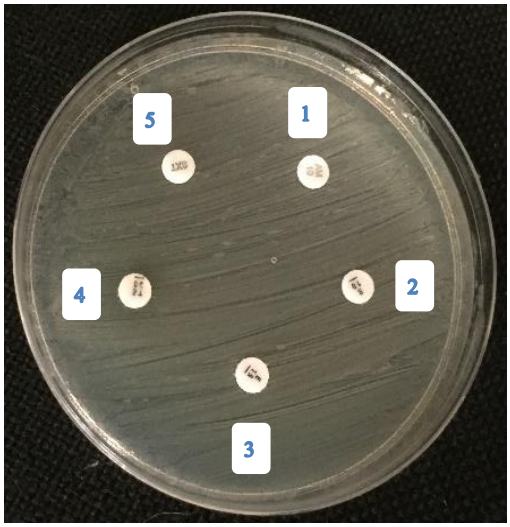


**Figure 1**. Patterns of Escherichia coli resistance to several classes of antibiotics (1) Ampicillin, (2) Streptomycin, (3) Erythromicin, (4) Tetracycline, and (5) Sulfamethoxazole-Trimethropin

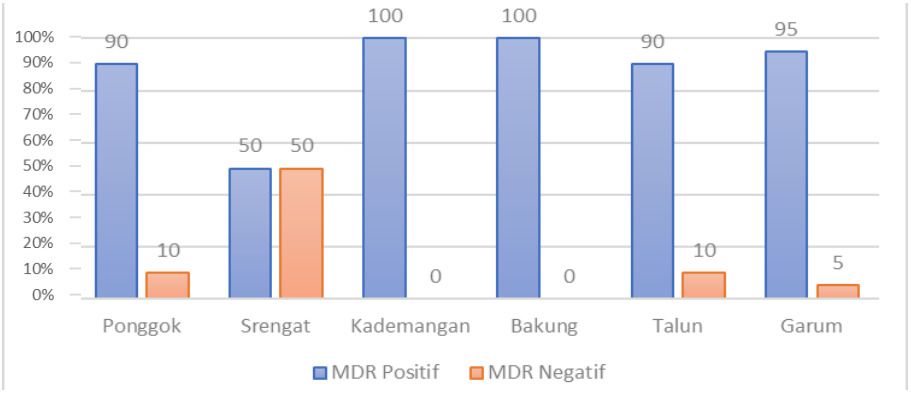


**Figure 2**. Diagram of Multi-Drug Resistant (MDR) of Escherichia coli on Broiler chickens in Blitar district

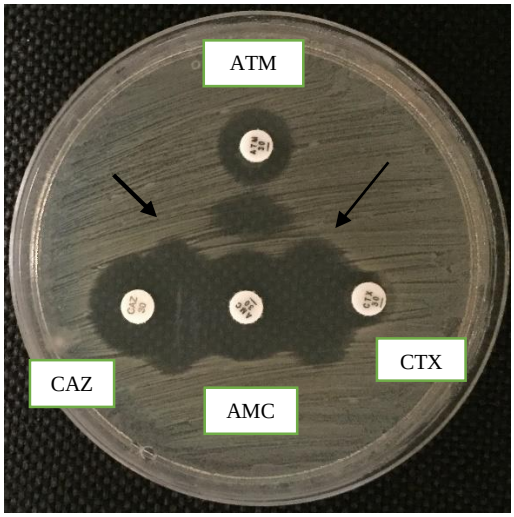


**Figure 3**. Positive ESBL-producing Escherichia coli with Double Disc Synergy Test (DDST) Method. Description: black arrow: synergy formed, ATM: Aztreonam, CAZ: Ceftazidime, AMC: Amoxycillin clavulanic, and CTX: Cefotaxime.

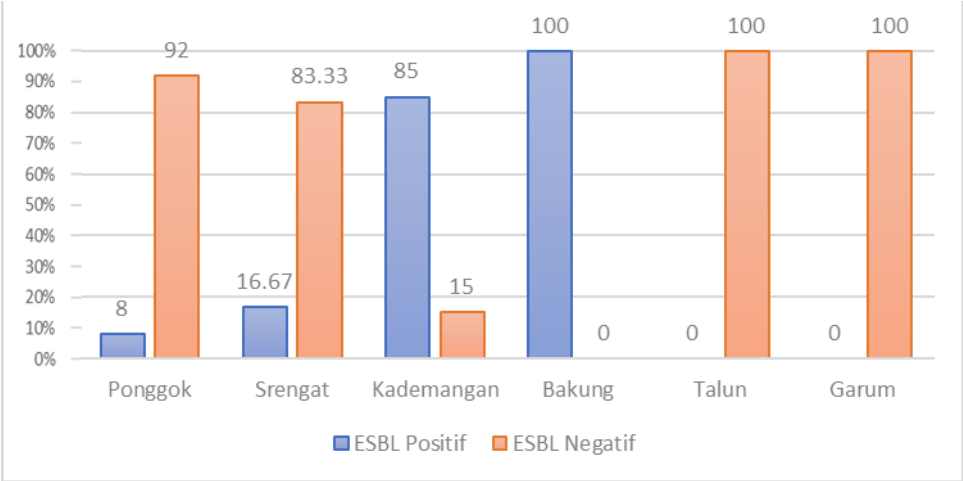


**Figure 4**. Diagram of ESBL-producing Escherichia coli on Broiler chickens in Blitar District

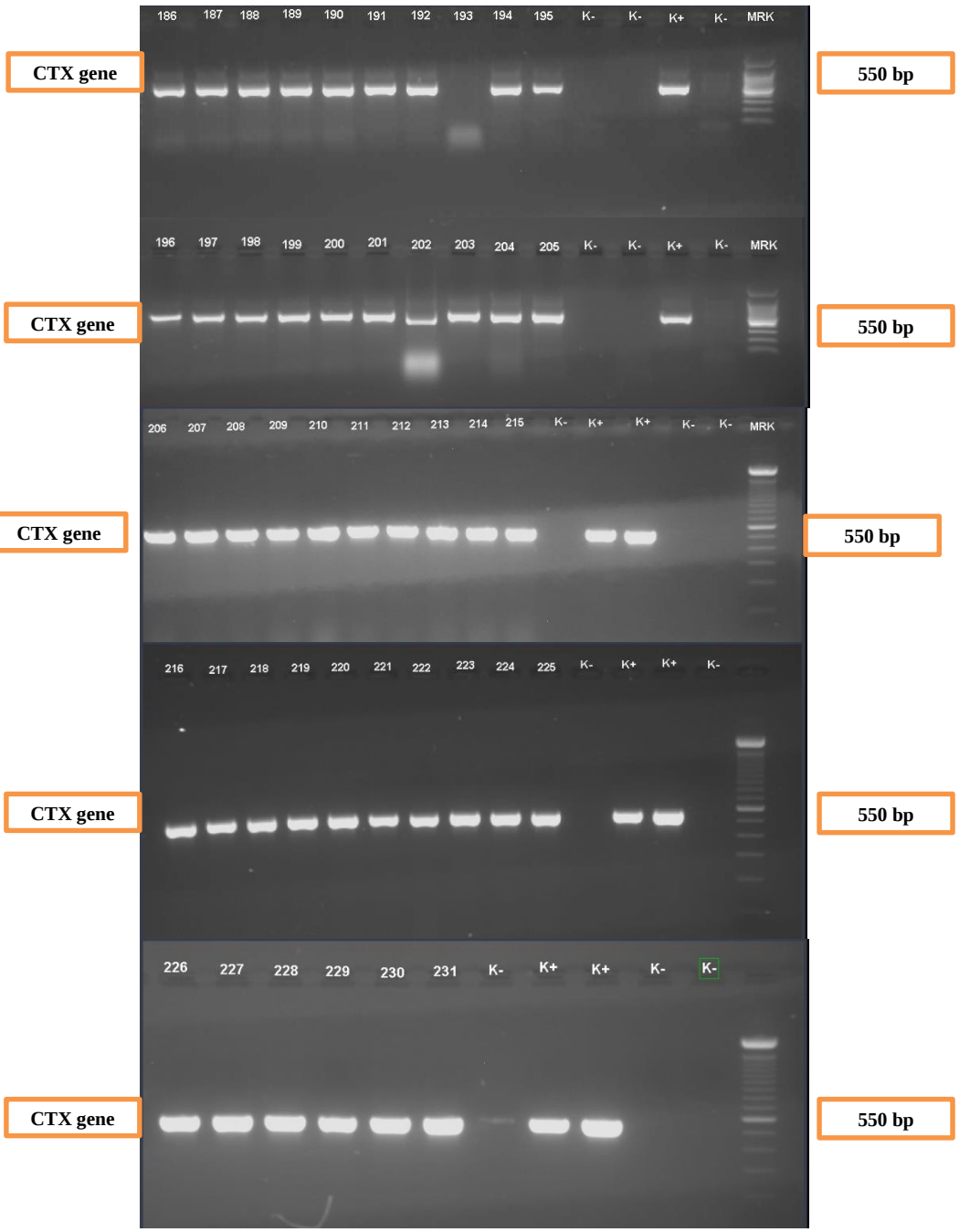


**Figure 5**. Molecular identification of ESBL-producing Escherichia coli of CTX gene and sample code 193 there was negative for CTX gene.