Antibiotic resistance pattern of Extended-Spectrum β-Lactamase (ESBL) producing *Escherichia coli* isolated from broiler farm environment in Pasuruan district, Indonesia

SHEILA MARTY YANESTRIA¹, FIDI NUR AINI EKA PUJI DAMEANTI¹, BETI GISTAWATI MUSAYANNAH², JUNIANTO WIKA ADI PRATAMA³, ADIANA MUTAMSARI WITANINGRUM⁴, MUSTOFA HELMI EFFENDI⁵*, EMMANUEL NNABUIKE UGO⁶

¹Doctoral Program in Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga. Jl. Mulyorejo, Kampus C FKH-UNAIR, Surabaya 60115, East Java, Indonesia
²Undergraduate Student in Faculty of Veterinary Medicine, Universitas Wijaya Kasuma. Jl. Dukuh Kupang XXV No.54, Surabaya 60225, East Java, Indonesia
³Department of Basic Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Wijaya Kasuma. Jl. Dukuh Kupang XXV No.54, Surabaya 60225, East Java, Indonesia
⁴Department of Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Airlangga. Jl. Mulyorejo, Kampus C FKH-UNAIR, Surabaya 60115, East Java, Indonesia. Tel./fax.: +62-31-5992785, *email: mhelmi@efendi@gmail.com
⁵Department of Applied Microbiology, Faculty of Science, Ebonyi State University. 480211, Abakaliki, Nigeria

Abstract. Yanestria SM, Dameanti FNAEP, Musayannah BG, Pratama JWA, Witaningrum AM, Effendi MH, Ugbo EN. 2022. Antibiotic resistance pattern of Extended-Spectrum β-Lactamase (ESBL) producing *Escherichia coli* isolated from broiler farm environment in Pasuruan district, Indonesia. Biodiversitas 23: 4460-4465. *Escherichia coli* is one of the bacteria that can be used as an indicator of environmental pollution. This bacterium has the ability to become an antimicrobial-resistant bacterium that impacts public health. The antimicrobial ability can develop into a bacterium that produces extended-spectrum β-lactamase (ESBL). The purpose of this study was to reveal the occurrence of extended-spectrum β-lactamase (ESBL) producing *E. coli* from a broiler farm environment in Pasuruan district and to describe the phenotypic pattern of *E. coli* producing ESBL that has been detected. A total of 175 samples were used in this study consisting of 115 samples of coop swabs and 65 samples of wastewater around the farm. The samples were isolated and identified to find *E. coli* by using different culture media viz. McConkey agar (MCA), eosin methylene blue agar (EMBA), Gram staining, indole test, methyl red Voges Proskauer (MR-VP), citrate, and triple sugar iron agar (TSIA). Detection of ESBL using the double disc synergy test (DDST) according to standard Clinical and Laboratory Standards Institute procedures and the VITEK®2 compact apparatus. The results of ESBL confirmation with DDST and VITEK®2 showed that 16 (9.14%) of the 175 environmental samples confirmed *E. coli* produced ESBL. The results of the VITEK®2 test also produced a phenotypic pattern of resistance properties of ESBL-producing *E. coli* and found 12 types of resistance patterns. The combination of “AM AMP ATM KZ CTX CRO GM CIP SXT” are the 2 most common resistance patterns (18.75%), while the other 10 resistance patterns occur at the same level (6.25%). The data presented here confirmed the presence of ESBL-producing *E. coli* in the farm environment, which can contribute to the dissemination of MDR bacteria in the environment if not monitored. Therefore, the presence of ESBL-producing *E. coli* in Pasuruan is worrisome since it can lead to an impact on human health.

Keywords: *Escherichia coli*, ESBL, public health, resistance patterns, VITEK®2 test

**INTRODUCTION**

Resistance is the ability of any bacteria to adapt to antibiotic exposure (Spellberg et al. 2013). This trait is a natural mechanism for survival. The main cause of resistance is the overuse of antibiotics in humans and animals (Niasono et al. 2019; Rahmahani et al. 2020). Bacterium show resistance to antibiotics if their growth cannot be inhibited antibiotics, even at maximum doses. A bacterium that carries antimicrobial resistance has a fatal effect on the patient. Deshpande et al. (2011) stated that antimicrobial resistance resulted in a prolonged illness, and increased risk of a longer period of hospitalization and death.

One form of antimicrobial resistance (AMR) is extended spectrum β-lactamase (ESBL). ESBL is a β-lactamase enzyme that can hydrolyze penicillins, first, second, and third-generation cephalosporins and aztreonam (except cephemycin and carbapenem) where their activity can be inhibited by clavulanic acid. The gene encoding ESBL is located on a plasmid that is easily transferred to another bacterium, resulting in the spread of resistance (Paterson and Banomo 2005). Various studies have shown that ESBL bacterium causes higher morbidity and mortality than non-ESBL bacterium (Nathisuwan et al. 2001; Paterson and Banomo 2005).

Resistance to ESBL-producing bacterium has been widely reported in Indonesia, including ESBL-producing
Escherichia coli originating from broiler farms (Effendi et al. 2021). Escherichia coli producing ESBL was found by detecting the CTX-M gene in broiler chicken feaces samples at the Bogor City Chicken Slaughter Center in as many as 12 of 200 samples (6.0%) (Lukman et al. 2016). ESBL-producing E. coli which was also found by detecting the CTX gene in broiler cloacal swab samples in Blitar City were 45 of the 160 samples examined (28.13%) (Wibisono et al. 2020a).

The presence of ESBL-producing E. coli in food-producing animals is associated with public health problems because spread from animals to humans can occur at any time (Masruroh et al. 2016). Resistant bacterium contained in feces can migrate around farms, poultry slaughterhouses and during meat processing (Santos et al. 2013). There are several studies that show a relationship between the transfer of the ESBL-producing E. coli gene from birds to humans who come into direct contact with these animals. In addition to direct zoonotic transfer, food of animal origin has the potential to be a risk factor for bacterium colonization or infection in humans (Widodo et al. 2020).

The occurrence of E. coli producing ESBL in broiler chickens, apart from being caused by the unwise and overuse of antibiotics on farms, can also occur due to the transfer of resistant material from the environment outside the cage into the cage environment. The environment around the cage can store various resistant materials that can transfer between bacteria (Niasono et al. 2016, Widodo et al. 2020). The environment is the main source of resistance, there is a relationship between humans, animals and the environment that allows the transfer of bacteria including mobile genetic elements (MGEs) between bacterium species (Woolhouse et al. 2015; Riwu et al. 2020). The spread of resistance to other organisms through MGEs (plasmids and transposons) is being accelerated and strengthened by agricultural and livestock activities as well as human waste that pollutes the environment (FAO 2018). Therefore, the purpose of this study was to reveal the occurrence of E. coli producing ESBL from a broiler farm environment in Pasuruan district and to describe the phenotypic profile of E. coli producing ESBL that has been detected.

MATERIALS AND METHODS

Sampling collection
Sampling was carried out in three sub-districts (Sukorejo Pandaan, and Kejayan) in Pasuruan district, Indonesia. A total of 175 samples were taken in this study which consisted of 115 samples of swab cages and 65 samples of wastewater around the farm. The swab sample of the cage is a swab from the wall of the chicken coop which was taken aseptically using a sterile cotton swab (Onemed, Indonesia). All swab samples were put into sterile tubes containing sterile buffered peptone water (HiMedia, India) during transportation. A sampling of wastewater is carried out in rivers or ditches closest to the farm. A sample of 50 mL of wastewater was put into sterile plastic bottles for transportation (Ibrahim et al. 2013; Samanta et al. 2014). All samples were then taken using a thermobox at a temperature of 4°C to the laboratory for further analysis (Yanestria et al. 2019).

Isolation and identification of Escherichia coli
The E. coli enrichment stage was carried out by inserting 1 mL of wastewater or a swab sample into 9 mL of Mac Conkey broth (HiMedia, India) in a test tube, then incubated at 43°C for 24 h (Damayanti and Purwantisari, 2020). Escherichia coli was isolated using selective eosin methylene blue agar (EMBA) media (Oxoid, England) and incubated at 37°C for 18-24 h (Putra et al. 2020). Identification of E. coli using Gram staining and biochemical tests IMVIC (Indol-motility, methyl red, Voges Proskauer, citrate) and TSIA (Triple sugar iron agar) (Effendi et al. 2019; Wibisono et al. 2021).

Antibiotic sensitivity testing
Antibiotic Sensitivity Testing assay was carried out by using disc diffusion antibiotic sensitivity test using Mueller Hinton agar (MHA) (HiMedia, India) in accordance with the Clinical and Laboratory Standards Institute standard procedures. The disc (Oxoid, England) used in this study consisted of 5 antimicrobial classes (AMC), namely fluoroquinolones (ciprofloxacin, nalidixic acid), macrolides (erythromycin), tetracyclines (tetracyclines), aminoglycosides (streptomycin, gentamicin), β-lactams (ampicillin, amoxicillin-clavulanate, aztreonam). Planting in plates was done by taking 1-2 colonies of E. coli isolates on EMBA media using loop inoculation, then put into physiological NaCl using 0.5 Mc Farland (1.5x10⁸ CFU/ml). By using a sterile swab, the suspension that has been made is then flattened on the surface of the MHA media. The plates were incubated at 35°C±2°C for 16-18 h under aerobic conditions. Interpretation of inhibitory results by measuring the diameter of the inhibition zone formed according to CLSI (CLSI 2020; Musa 2020; Putra et al. 2020). An isolate is defined as multidrug resistant if it is not sensitive to one or more antimicrobials in 3 different antimicrobial classes (Varga et al. 2019).

Confirmation of ESBL using Double Disc Synergy Test (DDST)
Isolates that were positive for multidrug resistance (MDR) were forwarded to the double disc synergy test (DDST). The assay was performed on Mueller Hinton Agar with three antibiotic discs (Oxoid, England) including ceftazidime (CAZ; 30 g), cefotaxime (CTX; 30 g), and Amoxycillin-clavulanic (AMC; 30 g) placed on the media in parallel. The aztreonam antibiotic disc (ATM, 30 g) was placed next to the three parallel discs. The cultures were incubated at 35°C±2°C for 16-18 hours. The test was considered positive when there was synergy and an increase in zone diameter of 5 mm for any of the antimicrobial agents tested in combination with clavulanate (Guo et al. 2019; CLSI 2020; Wibisono et al. 2021).
Confirmation of ESBL using VITEK®2

Isolates that were positive for ESBL in the DDST assay were forwarded to testing using the VITEK®2 compact (Biomerieux, USA) to confirm ESBL-producing E. coli and its resistance phenotypic profile. After isolation of the primary organism, handling is minimized in a simple standard inoculum. The inoculum is placed into the VITEK®2 cassette at the SMART CARRIER STATION™, where the VITEK®2 card and the sample are virtually connected. After the cassette is loaded, the incubation and read system regulates the incubation and reading of each card (Putra et al. 2020).

RESULTS AND DISCUSSION

The results of the isolation and identification of E. coli showed that 35.65% were positive for E. coli from 115 samples of swab cages and 64.62% were positive for E. coli from 65 samples of wastewater. About 47.43% of the total 175 samples were detected with E. coli (Figure 1). AMR testing using the diffusion method showed that 33.04% of 115 coop swab samples and 36.92% of 65 wastewater samples, a total of 34.86% of 175 environmental samples, detected the presence of multidrug resistance E. coli. The results of ESBL confirmation with DDST and VITEK®2 showed that 16 (9.14%) of the 175 environmental samples confirmed to be E. coli producing ESBL (Figures 3-2, 3, Table 1). The VITEK®2 test results also produced a phenotypic pattern of resistance properties of ESBL-producing E. coli (Table 2).

All ESBL-producing E. coli isolates (100%) were resistant to amoxicillin, ampicillin, cefazolin, cefotaxime and ceftiraxone, and very high resistance to aztreonam (87.5%) and ciprofloxacin (81.25%). To a lesser extent, resistance to gentamicin was about 62.5% and trimethoprim/sulfamethoxazole was about 56.25%. Low resistance rates were similar to the antibiotics ceftazidime (31.25%), ampicillin-sulbactam (25%) and lowest to cefepime (18.75%).

Multidrug resistance to three antimicrobial classes (AMC) was detected in all ESBL-producing E. coli isolates. The frequency profile of resistance to 4 and 5 AMC is 37.5% and 31.25%, respectively.

Based on the results of the study, 12 types of resistance patterns were found in E. coli producing ESBL (Table 2). The combination of “AM AMP ATM KZ CTX CRO CIP” and “AM AMP ATM KZ CTX CRO GM CIP SXT” were the 2 most common resistance profiles (18.75%), while the other 10 resistance profiles occurred at the same level (6.25%).

Table 1. Detection Results of Extended Spectrum β-Lactamase (ESBL) producing Escherichia coli from Environmental Samples

<table>
<thead>
<tr>
<th>Samples type</th>
<th>Sample size</th>
<th>Escherichia coli</th>
<th>MDR</th>
<th>DDST</th>
<th>ESBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coop Swabs</td>
<td>115</td>
<td>41</td>
<td>38</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Waste water</td>
<td>65</td>
<td>42</td>
<td>24</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>175</td>
<td>83</td>
<td>62</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

Note: MDR: Multidrug Resistance, DDST: Double Disc Synergy Test

Figure 1. A. Escherichia coli on eosin methylene blue agar plate, B. Microscopic of Escherichia coli

Figure 2. Positive result of double disc synergy test. Note: Green arrow indicates synergy of antibiotics

Figure 3. Results of detecting the amount of Extended Spectrum β-Lactamase (ESBL) producing Escherichia coli from environmental samples
Table 2. Results of ESBL-producing Escherichia coli Phenotypic Patterns using VITEK®2

<table>
<thead>
<tr>
<th>Antibiotics resistance pattern</th>
<th>No of AMC</th>
<th>Escherichia coli ESBL</th>
<th>Sample code</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM AMP ATM KZ CTX CRO CIP</td>
<td>3</td>
<td>3</td>
<td>S 26.1; S 42.2; A 15.1</td>
</tr>
<tr>
<td>AM AMP ATM SAM KZ CTX CRO GM</td>
<td>3</td>
<td>1</td>
<td>S 1.3</td>
</tr>
<tr>
<td>AM AMP ATM KZ CTX CRO FEP GM</td>
<td>3</td>
<td>1</td>
<td>S 10.3</td>
</tr>
<tr>
<td>AM AMP KZ CTX CRO GM CIP</td>
<td>4</td>
<td>1</td>
<td>A 38.1</td>
</tr>
<tr>
<td>AM AMP ATM KZ CTX CRO CIP SXT</td>
<td>4</td>
<td>1</td>
<td>A 10.3</td>
</tr>
<tr>
<td>AM AMP ATM SAM KZ CTX CAZ CRO CIP SXT</td>
<td>4</td>
<td>1</td>
<td>S 2.3</td>
</tr>
<tr>
<td>AM AMP ATM SAM KZ CTX CAZ CRO GM CIP</td>
<td>4</td>
<td>1</td>
<td>S 1.2</td>
</tr>
<tr>
<td>AM AMP ATM SAM KZ CTX CAZ CRO CIP SXT</td>
<td>4</td>
<td>1</td>
<td>S 19.1</td>
</tr>
<tr>
<td>AM AMP ATM SAM KZ CTX CAZ CRO GM SXT</td>
<td>4</td>
<td>1</td>
<td>S 1.1</td>
</tr>
<tr>
<td>AM AMP ATM KZ CTX CRO GM CIP SXT</td>
<td>5</td>
<td>3</td>
<td>S 10.2; S 12.1; S 44.2</td>
</tr>
<tr>
<td>AM AMP ATM KZ CTX CRO FEP GM CIP SXT</td>
<td>5</td>
<td>1</td>
<td>S 18.4</td>
</tr>
<tr>
<td>AM AMP ATM KZ CTX CRO FEP GM CIP SXT</td>
<td>5</td>
<td>1</td>
<td>S 52.2</td>
</tr>
</tbody>
</table>


Discussion

In Indonesia, information about E. coli producing ESBL in chicken farms is still limited. This study showed that the prevalence of ESBL-producing E. coli in broiler farms is around 9.14%. This prevalence can be considered low as compared to that reported by researchers from other countries. Research in Thailand, Vietnam, and Pakistan, both in Asia, showed the prevalence of E. coli ESBL in the poultry environment was 43.8%, 42.6%, and 14% (Ueda et al. 2015; Rahman et al. 2018; Rodroo et al. 2015). In other countries, Washington State has a prevalence of 11% and Spain has a prevalence of 20.7% (Shah et al. 2019; Alvarez et al. 2022). However, the prevalence in this study was considered higher when compared to studies from Nigeria with a prevalence of 4.6% (Aworh et al. 2020).

All ESBL-producing E. coli isolates (100%) were MDR. This is consistent with other studies in Thailand and Nigeria that all ESBL-producing E. coli originating from the polluting environment have MDR properties (Tansawai et al. 2019; Aworh et al. 2020). The problem of MDR is exacerbated by the ability of the bacteria to transfer genetic material that carries resistance traits from one bacterium to another vertically through genetic mutations and horizontally through conjugation, transduction and transformation. MDR occurs when over antibiotics are used, the greater the selective pressure on the evolution and proliferation of resistant bacterium strains to defend themselves, resulting in vertical resistance from genetic mutations and horizontal resistance from the exchange of resistant gene material against various types of different antibiotic resistance mechanisms. Multidrug resistance is a common occurrence in ESBL-producing bacterium (Gregova et al. 2012; Effendi et al. 2021).

In this study, the resistance pattern "AM AMP ATM KZ CTX CRO GM CIP SXT" is one of the most frequent resistance phenotype profiles of ESBL-producing E. coli (3 out of 16 ESBL-producing E. coli isolates). This incident is similar to the results of another study in Indonesia (Bilitar District) which showed this resistance pattern was also the most common (3 out of 10 ESBL-producing E. coli isolates), but the sample used was a cloacal swab (Wibisono et al. 2020b). There is a similar pattern of resistance although different types of samples, this study used environmental samples of poultry, while the other used a poultry cloacal swab. This is due to the high use of this antibiotic in the poultry sector, besides it can be related that E. coli that produces ESBL that is spread in the chicken farm environment comes from chicken feces.

The presence of ESBL bacterium in the poultry sector occurs due to the use of antibiotics as treatment, metaphylactic, prophylactic and growth promoters. The prohibition on the use of antibiotics as growth promoters has been compensated by the increasing use as metaphylactic and prophylactic in commercial livestock (Woolhouse et al. 2015; Wongsvan et al. 2018, Permatasari et al. 2020). Antibiotics are often added to water or animal feed including broilers as a pragmatic solution because they are raised in groups making it difficult to isolate and treat only the infected. In addition, efforts to isolate broiler chickens can stress animals (Singer et al. 2016).

The percentage of the drug dose metabolized or absorbed by individual animals ranges from 10-80%, depending on the species and the drug used, the rest is excreted as the active compound through urine and feces into the environment. Waste from animals treated with antibiotics contains antibiotics and resistant bacteria (FAO 2018). Several studies in Indonesia have shown that E. coli producing ESBL is detected in broiler chicken feces, referring to the studies of Lukman et al. (2016) and Masuroh et al. (2016).

The release of antibiotics into the environment also coincides with the release of antibiotic resistance genes (ARGs) (Hu et al. 2013; Chang et al. 2015; Newton et al. 2015). Co-location of antibiotics and ARGs in the environment can lead to novel combinations of AMR that can be shared between microorganisms by horizontal gene transfer (HGT) on mobile genetic elements (MGEs), such as plasmids, thereby increasing the prevalence and combination of multiple drug resistances in the microbial
community (Xu et al. al., 2015). The competitive and chemically challenging environment provides favorable conditions for amplifying resistance genes and creating new resistance genes or genome sets (Zhang and Zhang 2011).

ESBL-producing bacterium in the environment can spread from animals to humans and have the potential to cause zoonotic diseases. Spread can be through direct contact with a polluted environment and consumption of contaminated meat. Chickens that have E. coli that produce ESBL in their digestive tract or come from a cage environment that is contaminated with ESBL-producing E. coli, it is possible that their meat is contaminated with this bacterium if during slaughter and handling of meat it is not hygienic. In Indonesia, there have been studies have shown ESBL-producing E. coli contamination in broiler chicken meat. Mu’arofah et al. (2020) reported a prevalence of 30% in samples from traditional markets in East Surabaya. Widhi and Saputra (2021) reported a prevalence of 71.4% in a sample from the Purwokerto City Market. Wardhana et al. (2020) reported a prevalence of 90.03% in a sample from the Surabaya Traditional Market.

Chicken meat contaminated with ESBL bacterium has the potential to spread the bacterium to humans (Harijanti et al. 2020). Foods of animal origin have a higher risk making them difficult to handle and control. In the era of global trade, the presence of ESBL in products of animal origin is a threat that must be taken seriously (Calisti et al. 2013). Health problems caused by these resistant bacterium infections can include medical costs, limited treatment options for patients, longer hospital stays, and even death. It is known that E. coli is harmful to health because it produces a toxin, like the Shiga toxin (Ansharieta et al. 2015). Origin and proliferation of multiple-drug resistance in bacterial pathogens. Microbiol Mol Biol Rev 79: 101-116. DOI: 10.1128/MMBR.00039-14.

In conclusion, present studies indicated the presence of 16 (9.14%) of the 175 environmental samples that confirmed ESBL-producing E. coli in Pasuruan, Indonesia. The results of the VITEK®2 test also produced a phenotypic pattern of resistance properties of ESBL-producing E. coli and found 12 types of resistance patterns. VITEK®2 test results further explain the resistance pattern of ESBL producing E. coli. The presence of ESBL-producing E. coli in Pasuruan is worrisome since it can lead to an impact on human health due to eating contaminated chicken meat and the use of water contaminated by wastewater from poultry farms. There is an urgent need for constant and proper monitoring of the use of antimicrobial agents on chicken farms by veterinarians.

ACKNOWLEDGMENTS

This article was supported in part by the Penelitian Pasca Sarjana - Penelitian Disertasii Doktor funding from PENELITIAN DRTPM KEMENDEKUBRISTEK TAHUN 2022, with grant number from Universitas Airlangga: 905/UN3.18/P/2022.

REFERENCES


CNSL. 2020. Performance standards for antimicrobial susceptibility testing. 30th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute


Penelitian DRTPM Kemendikbudristek Tahun 2022 with grant number from Universitas Airlangga: 905/UN3.18/P/2022


