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Molecular detection of *bla*$_{TEM}$ gene for encoding extended spectrum beta-lactamase (ESBL) on *Escherichia coli* isolated from deer feces in Indonesia

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ABSTRACT

Antibiotic resistance is a serious global health threat and broad-spectrum beta-lactamase- producing Enterobactericeae (ESBL) are major contributors. This research focuses on the presence of *Escherichia coli* as a producer of ESBL in the fresh feces of deer. This study aimed to gain deeper insight into ESBL sourced from deer. 129 fresh stool samples were collected. A total of 89 samples came from the East Java region and 40 samples came from the East Nusa Tenggara region. Identification was carried out which was then followed by PCR. We found 9 positive samples of ESBL-producing *E. coli* containing the *bla*$_{TEM}$ gene as the ESBL encoding gene. 33 samples (25.28%) were positive for *E. coli*. 8 isolates (24.24%) were multidrug-resistant, East Java (6 isolates) and East Nusa Tenggara (2 isolates). All isolates of multidrug-resistant *E. coli* 7 isolates were ESBL positive (87.5%) and contained the blaTEM gene. The presence of MDR and ESBL-producing *E. coli* isolates have been confirmed in deer. Deer should be considered as a source of MDR and ESBL transmission for public health.

Introduction

Antibiotic resistance is an important problem in animal and human health. Bacteria that naturally have antibiotic-resistance genes can transfer these genes to other bacteria. In addition, bacteria are also able to produce enzymes that work to inhibit the performance of antibiotics (Aidara-Kane *et al*., 2013). The development of antibiotic resistance has spread worldwide. Beta-lactam antibiotic resistance generally occurs in Gram-negative bacteria. ESBL-producing bacteria, such as *Escherichia coli* can be isolated from various food-producing animals which are known to be reservoirs for ESBL-producing *E. coli* (Schmid *et al*., 2013; Anshariet*a et al*., 2021). The gene that forms the ESBL enzyme was discovered after the discovery of the extended-spectrum cephalosporin and was first introduced in Europe in the 1980s. ESBL-forming genes can be found in *E. coli* isolates in various countries.

In addition, ESBL is a mutant form of TEM-1, TEM-2, and SHV-1. Often the genes that makeup ESBL are changed from their original form by changing only one or more amino acid sequences. This ESBL enzyme has been spread in various organisms (Tyasningsih *et al*., 2022; Yanestria *et al*., 2022). These enzymes can not only hydrolyze penicillin, but also the newest antibiotics, namely group 3 cephalosporins and monobactams. ESBL-producing *E. coli* can be found in humans, farm animals, and wildlife, in gastrointestinal tract tissue, and in infected urine tissue (Schaufler *et al*., 2015; Putra *et al*., 2020). Environmental contamination by ESBL bacteria has been widely reported, especially in developed and developing countries. Wastewater has a high potential for spreading this ESBL infection, especially hospital waste which mostly contains ESBL-producing *E. coli* (Yanestria *et al*., 2022).

Not only that, wildlife, especially birds, can contribute to the spread of these ESBL bacteria by migrating freely from one area to another, between urban and agricultural land, which plays a role in the spread of ESBL bacteria, especially in areas that are ecologically poor or polluted (Homeier-Bachmann *et al*., 2022). The spread of infectious diseases through international travel has been widely reported, for example, tourists from developed countries traveling to endemic countries or countries with poor sanitation, and low medical costs, can also play a role in the import of bacteria that are resistant to several antibiotics, including ESBL bacteria after returning to their country of origin carrying (carriage) (Doi

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et al., 2017).

The presence of wild animals in the environment is not in direct contact with antibacterials. It is possible that their wild animals may be exposed to antibacterial residues by foraging and drinking in contaminated environments with associated anthropogenic activities (Homeier-Bachmann *et al*., 2021). The results of several previous studies showed that the presence of wildlife living near humans and close to agricultural areas showed the incidence of cases caused by antimicrobial resistance showed higher results compared to the results of those living in environments far from humans (Torres *et al*., 2020). The diversity of wildlife, the pattern of food consumed, and also the high tolerance of animals to activities and their proximity to the environment inhabited by humans are things that can cause antimicrobial resistance and antimicrobial residues, therefore this causes wildlife species to be believed as species potential reservoir for antimicrobial resistance in wildlife (Torres *et al*., 2020).

There are many questions regarding the role of wildlife, for example, in the context of the incidence of antimicrobial resistance cases in the wild as well as cases of human-associated infections (Dolejska and Literak, 2019). Previously published studies stated that the incident involved wild animals in the forest, and the most common bacterial species found was *E. coli* (Torres *et al*., 2020). This study aimed to analyze ESBL-producing *E. coli* collected from deer as wildlife in a limited investigated area in Indonesia. We specifically discuss the molecular detection of ESBL-producing *E. coli*. Feces from wild deer were chosen to reveal the presence of *E. coli* as a producer of ESBL from wild animals so that its resistance to beta-lactam antibiotics can be determined.

Materials and methods

Ethical approval

The deer feces samples used in this study were taken from several zoos in Indonesia, so ethical approval was not required. Samples were taken accord-ing to standard collection procedures.

Research design

A total of 129 fresh fecal samples were taken from deer breeding sites in the provinces of East Java (89 samples) and East Nusa Tenggara (40 samples). Fresh feces of deer were weighed as much as 25 g. The sample was homogenized in a stomacher by adding a 0.1% BPW (Buffered Peptone Water) solution with a ratio of 1:10. The next step is to cultivate the sample by taking 1 round loop and then streaking it on Mac Conkey Agar (MHA) and incubating at 37°C, 24 hours. The colonies suspected of being *E. coli* were also tested for KOH, Gram staining, and biochemical tests (indole, methyl red, Voges Proskauer, sulfide indole motility (SIM), Simmons citrate test (SCA), and carbohydrate fermentation tests (glucose, lactose, mannitol, maltose, sucrose) and incubated at 37°C for 24 hours, the isolates obtained were then identified (Effendi *et al*., 2019).

Antibiotics sensitivity test

Isolates that have been isolated and identified are tested for susceptibility to antibiotics using the Kirby- Bauer disk diffusion test method, by making a suspension of bacterial colonies whose turbidity is equated with the standard 0.5 McFarland 1 which is equivalent to the concentration of bacteria 1.5 x 106 CFU/ml. Next, a sterile cotton swab is dipped in bacterial suspension and smeared on Mueller Hinton Agar (MHA) media. The media was allowed to dry for five minutes then antibiotic discs (Tetracycline (30 µg), Streptomycin (10 µg), Trimethropim (5 µg), Chloramphenicol (30 µg), Aztreonam (30 µg), were affixed to MHA and gently pressed and incubated at 37°C for 24 hours. The diameter of the inhibition zone formed around the antibiotic disc was measured with a ruler in millimeters. The interpretation of the inhibition zone measurement was based on the Clinical and laboratory standards institute (CLSI) (CLSI, 2018).

Double Disc Synergy Test

Confirmation of ESBL in the study was carried out using the double disc synergy test method. following the methods established by the British Society for Antimicrobial Chemotherapy Pure cultures were prepared in suspension with turbidity equivalent to 0.5 McFarland. The culture is taken using a sterile cotton swab and spread on the surface of Mueller Hinton agar (MHA), and allowed to stand for ± 5 minutes. Disc paper containing antibiotics (ceftazidime 30 µg, amoxiclav 20 + 10 µg, and cefotaxime 30 µg) was placed on top of MHA, which had been spread in pure culture, with a distance of 25-30 mm. The culture was then incubated at 35°C for 24 hours. ESBL confirmation was carried out with controls, namely *E. coli* ATCC 25922 as positive controls and K. pneumonia ATCC 700603 as negative controls.

PCR Analysis

The DNA extraction procedure was carried out using the QIAamp DNA Mini Kit (50) from QIAGEN following the manufacturer's instructions. A total of 1ose of bacterial colonies + 180 µl of ATL buffer was inserted into a 1.5 ml microtube, and vortexed. Add 20 µl proteinase K + 200 µl buffer AL, incubate 56°C, for 1 hour and 70°C, for 10 minutes. The mixture was + 200 µl absolute ethanol, vortexed for 15 seconds, then transferred to a filtered tube (mini column) and centrifuged at 8000 rpm for 1 minute. The supernatant was discarded and the filter was rinsed with 500 µl buffer AW1, centrifuged at 8000 rpm for 1 min, and the supernatant was discarded. This flushing process is repeated in the same way using the AW2 buffer. Then centrifuged at 14000 rpm for 1 minute. Finally, the filter was placed in a new tube, rinsed with 200 µl of buffer AE and centrifuged at 8000 rpm for 1 minute. The filter was removed, and the supernatant was stored at -20°C, as a DNA template (Ahmed and Shimamoto, 2015).

bla_{TEM} gene amplification of E. coli

The amplification process uses a reagent mix with a final volume of 35 µl consisting of 25 µl Go Taq Green; 1 µl bla_{TEM} forward primer; 1 µl bla_{TEM} reverse primer; 6 µl RNAse FW and 2 µl DNA template (Table 1). The amplification process uses a thermal cycler (Biorad) machine, starting with initial incubation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95ºC for 1 minute, annealing at 56°C for 1.5 minutes, elongation at 95°C for 1 minute and final extension at 95°C for 10 minutes (Abrar *et al*., 2019).

Table 1. Primer used in research.

Primer	Sequence (5' – 3')	Target Gene	Base pair	Reference
TEM-F	ATA AAA TTC TTG AAG ACG AAA	bla_{TEM}	1086	Yao *et al*. (2007)
TEM-R	GAC AGT TAC CAA TGC TTA ATC			

Results

On EMBA media, separate metallic green colonies were obtained. It can be assumed that metallic green colonies that grow on EMBA media are *E. coli*. These bacteria can form a metallic green color because they can ferment lactose and Methylene blue, which can be seen in Figure 1. In the DDST on *E. coli* isolates from deer feces samples, seven positive *E. coli* isolates that had been tested were positive for ESBL (Figure 2).

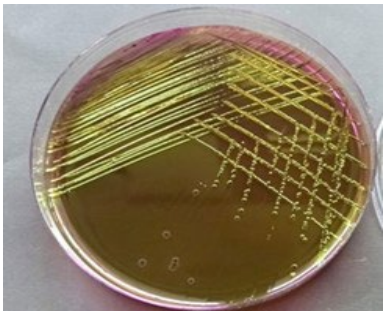

Fig. 1. *E. coli* isolates onto Eosin Methylene Blue Agar.

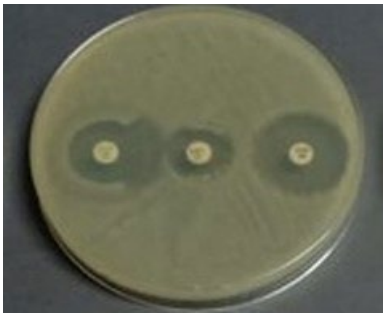

Fig. 2. Confirmed ESBL-producing *E. coli* by Double Disc Synergy Test

Based on Table 2, it can be seen from the 7 isolates that were tested by PCR using a special primer for the bla_{TEM} gene, all isolates (100%) showed positive PCR results, which means that the pathogenic bacteria *E. coli* isolates produce ESBL. More details can be seen in Figure 3 where the results obtained are in the form of DNA bands (DNA bands) at the 1086 bp position in the isolate samples of NTT2, NTT4, BBS6, BBS22, MRS2, MRS3, and MRK10. and compared with the positive control (K+) and negative control (K-) DNA bands (Figure 3).

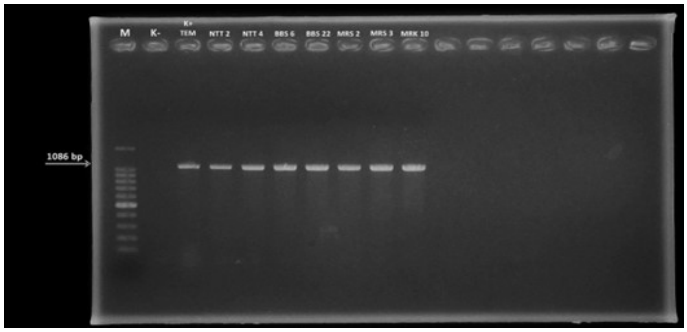

Table 2. Prevalence of extended-spectrum beta-lactamase (ESBL) producing *E. coli* from fresh deer feces.

Discussion

In this study, the prevalence of ESBL-producing *E. coli* sourced from fresh deer feces was significantly lower than in previously reported studies. It also differs in sample size and method used so this limits comparability. However, this match can only be confirmed for samples from 2020. Within the framework of the German National Zoonoses Monitoring Program, samples from roe deer were collected nationally in 2016 and 2017, respectively. Selective isolation of 2.3% ESBL-producing *E. coli* (13 of 573) from roe deer (Plaza-Rodríguez *et al*., 2020). Similar results from previous studies have been reported in the study by Holtmann *et al.* (2021). They collected stool samples in 22 different hunting areas in Germany between late 2014 and early 2015 and found 5.1% of ESBL-positive *E. coli* samples (19/375) (either $bla_{CTX-M-1}$ or bla_{SHV-12}).

In 2012-2013 in Poland, a study that included a total of 657 fecal samples, collected over two winters, came from a game that was shot during 46 hunts in the forest (2012–2013, n = 7 and 2013–2014, respectively). n = 39). The hunt took place in 37 forests spread across Poland (332 wild boars, 225 red deer, 76 roe deer, and 24 European fallow deer). 11 samples were found to be resistant to cefotaxime. Nine of them were taken from wild boar (2.7%, range 1.0-4.5%), and red deer (one isolate each) (Wasyl *et al*., 2018). A markedly higher prevalence estimate of the ESBL/AmpC-producing *E. coli* of 15.96% was mentioned in a study from southern Europe with a total of 1504 stool samples sourced from wild boars. The research has been carried out in northern Italy and consists of four hunting areas with an area of about 1,800 km^2. The sample was successfully collected in three consecutive hunting years between 2018 and 2020 (Formenti *et al*., 2021).

This is particularly useful for future comparisons of prevalence estimates for ESBL-*E. coli* is sourced from wildlife and will greatly help assess the AMR burden on wildlife with better yields. habitat, contact between wild animals with other animals such as livestock and humans will increase, which then results in an increase and exchange of antibiotic resistance both in the context of bacterial isolates but also changes in bacterial genetic elements. Previous studies have mentioned that wild animals living close to human populations have the potential to carry higher levels of AMR and MDR (Sousa *et al*., 2014; Plaza-Rodríguez *et al*., 2020). Of course, this can happen through the environment such as the water environment or contamination that occurs on agricultural land (Thaner *et al*., 2016; Homeier-Bachmann *et al*., 2021).

A recent study published also has a similar conclusion where the authors and researchers mention that wildlife carriers of antimicrobial resistance present in the environment can be transferred to pathogens to humans where previously this was very rare (Parkhill, 2022). The presence of *E. coli* ESBL bacteria in animal feces samples can pose a risk of contamination to the environment. *E. coli* is a bacterium that contaminates the environment and has the potential to cause health risks to animals and humans although the level of risk is difficult to quantify (Navab-Daneshmand *et al*., 2018). Infection with ESBL-producing bacteria through consumption of food of animal origin can also cause limited options in the treatment of infected patients. This situation can not only extend the treatment period, but also increase the cost of trand eatment, increase the incidence of disease, and death (Khosbayar *et al*., 2013).

The incidence of ESBL-producing *E. coli* that spread throughout the world has created problems in the health care system. The increasing prevalence of ESBL-producing pathogens and their evolution is due to the increasing frequency of administration of drugs such as penicillins, cephalosporins, monobactams, and carbapenems (Cheaito and Matar, 2014). The ESBL gene found in *E. coli* initially appeared by a plasmid- mediated gene mutation, namely the bla gene with type SHV and TEM. Then a new group from ESBL emerged, namely CTX-M (Cheaito and Matar, 2014). Food-producing animals are known as reservoirs for producing bacteria. Deer as one of the food-producing animals that can be obtained through illegal hunting to be used as food has the potential to become a reservoir for *E. coli*-producing ESBL. These ESBL-producing bacteria can spread from animals to humans and have the potential to cause zoonotic diseases. Spread can be through various routes, such as through the consumption of contaminated meat, through an environment contaminated with feces containing ESBL-producing *E. coli*, and also through contact with patients or individuals infected with ESBL- producing bacteria (Reich *et al*.. 2013; Haenni *et al*., 2014).

E. coli as an ESBL producer is also associated with resistance to other types of antibiotics, so this type of bacteria is referred to as multidrug resistance. Multidrug resistance is a phenomenon that often occurs in ESBL-producing bacteria. This is supported by Latifi *et al.* (2021) who says that the genes encoding resistance enzymes such as AME (Aminogly-

Table 2. Prevalence of extended-spectrum beta-lactamase (ESBL) producing E. coli from fresh deer feces

Sample origin	Sample size	*E. coli*	MDR	ESBL	bla_{TEM}
East Java	89	22 (24.7%)	6 (27.27%)	5 (83.33%)	5 (83.33%)
East Nusa Tenggara	40	11 (27.5%)	2 (18.18%)	2 (100%)	2 (100%)
Total	129	33 (25.58%)	8 (24.24%)	7 (87.5%)	7 (100%)

coside Modifying Enzyme) and ESBL are often found in bacterial plasmids. One of the main causes of the increasing prevalence of bacteria that are resistant to both beta-lactam and aminoglycoside antibiotics is gene transfer that occurs in genetic elements such as plasmids, integrons, and transposons (Allocati *et al*., 2013). Furthermore, the combination of several resistance genes causes bacteria to be resistant to most classes of antibiotics (Allocati *et al*., 2013).

The resistance caused by *E. coli* ESBL has an impact on the economy by reducing the productivity of infected animals, mortality, and medical costs throughout the livestock sector (Ibrahim *et al*., 2019). animals can act as reservoirs of pathogenic *E. coli* (El-Sawah *et al*., 2018). Animal feces become a source of *E. coli* transmission which can then be spread to the environment. *E. coli* ESBL is a pathogenic strain and can be transmitted to humans not only through food and drink contaminated with feces but also from the contaminated environment (Luna-Guevara *et al*., 2019). with antibiotics can control and prevent infectious diseases in animals (Schwarz *et al*., 2004). However, inappropriate administration of antibiotics can also cause bacteria to become resistant (Enne *et al*., 2014; Wibisono *et al*., 2021).

Bacterial plasmids are known to be able to accommodate resistance genes and spread resistance genes to bacteria, this is the origin and initial cause of resistance (Ramírez-Castillo *et al*., 2018). Various resistance genes can accumulate in bacterial plasmids, usually in the R (resistance) plasmid which is the reason for finding bacterial isolates that are resistant to various other antibiotics and then able to create new gene sequences (Nikaido, 2009). Multidrug-resistant *E. coli* isolates are responsible for a range of infections with high severity and difficult to treat, these are becoming very common in many countries. case study In Canada regarding infections that occur in the urinary tract due to bacteria, 60% of them are cases of multidrug-resistant *E. coli* infection. Consumption of raw or undercooked food of animal origin, travel habits between regions, and contact with reservoir animals are associated with an increased risk of urinary tract infections caused by MDR *E. coli* (Ukah *et al*., 2017).

In addition to being a reservoir of various antibiotic resistance genes, *E. coli* bacteria is also a reservoir of beta-lactam resistance encoding beta-lactamase genes (Effendi *et al*., 2021). Enterobacteriaceae produce ESBL enzymes that can hydrolyze penicillins and third-generation cephalosporins, monobactams, and other antibiotics, except for carbapenems (meropenem, imipenem, and ertapenem) (Pitout, 2012). This ESBL enzyme is mainly encoded by several specific encoding genes such as bla_{SHV}, bla_{CTX-M}, and bla_{TEM} genes (Bush, 2013; Wibisono *et al*., 2020). Many sources of exposure have the potential to transmit ESBL-producing *E. coli*, making epidemiological investigations very difficult. the horizontal gene transfer from bacteria resulted in a wider distribution of ESBL-encoding genes between various bacterial species.

Interactions occur at the microbial level in humans and animals, especially between commensal bacteria and pathogenic bacteria, facultative bacteria, and obligate bacteria in the same environment. 'One Health' is needed to understand and identify the possibility of preventing the spread of ESBL coding genes and infection in humans, through an integrative approach (Calistri *et al*., 2013). The application of the concept of One Health integration to accelerate disease prevention and prediction as an effort to control ESBL-producing *E. coli* (Wendt *et al*., 2015). Approaches to many sectors in of medical treatment in the field of veterinary medicine, medicine, and animal food production, can create cooperation in controlling the growth of antibiotic-resistant *E. coli*, for the benefit of public health (Economou and Gousia, 2015).

The importance of continuous monitoring of global resistance levels is very important for clinicians to support treatment choices and especially for patients receiving antibiotic therapy this is necessary to minimize contamination and reduce the *E. coli* transmission of antibiotic resistance (Wibisono *et al*., 2020). Source of attribution, which is on the microbial data subtype can help to understand and determine the causes of resistance in humans. This methodology is used to draw comparisons between genetic conclusions bacterial profiles present in various sources with which found in humans, noting similarities Such indices exist among the various isolates and cannot be based solely on sequence types, plasmid families and only ESBL genes, especially when the isolates are not spatially related. Sequencing the entire genome can help provide excellent information, but in addition, information about virulence is also needed and resistance gene content. Scientific information like this can help to trace and identify the source of human infection based on bacterial subtypes, distributed in various sources (Riwu *et al*., 2020).

Conclusion

Molecular identification of the bla_{TEM} gene found in fresh feces of deer from areas in East Java and Nusa Tenggara, Indonesia was used to identify *E. coli* that produce ESBL. The results showed that *E. coli* sourced from fresh deer feces produced ESBL with a relatively low prevalence. ESBL-producing *E. coli* shows potential for spread and poses a threat to public health.

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Conflict of interest

The authors have declared no conflict of interest.

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