Open Veterinary Journal, (2024), Vol. 14(2): 699-706
ISSN: 2226-4485 (Print)
ISSN: 2218-6050 (Online)

Original Research
DOI: *10.5455/OVJ.2024.v14.i2.10*

Submitted: 23/11/2023 Accepted: 27/01/2024 Published: 29/02/2024

Molecular detection of extended-spectrum β-lactamase-producing *Escherichia coli* from bat caves on Lombok Island

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ABSTRACT

Background: The discovery of antibiotic-resistant Enterobacteriaceae bacteria in wild animals is an indication of their potential for wildlife as a reservoir. Bats are natural reservoir hosts and a source of infection for several microorganisms and have the potential to become vectors for the spread of zoonotic diseases.

Aim: A study was conducted based on these characteristics to identify and detect the *bla*TEM gene in *Eschericia coli* isolated from bat excrements in Tanjung Ringgit Cave, East Lombok.

Methods: Bat fecal samples were firstly inoculated onto eosin methylene blue agar media. Recovered bacterial isolates were further characterized using standard microbiological techniques. Antimicrobial susceptibility testing was done using the Kirby-Bauer disc diffusion method. *bla*TEM gene detection was carried out using polymerase chain reaction (PCR).

Results: Out of the 150 bat fecal samples obtained from Tanjung Ringgit cave, Lombok Island, Indonesia, 56 (37%) were positive for *E. coli*. Eight (8) out of the 56 *E. coli* isolates that underwent antimicrobial susceptibility testing using the disc diffusion method were confirmed to be multidrug-resistant as they exhibited resistance to at least three different classes of antibiotics. Out of the eight (8) multidrug resistance *E. coli* isolates recovered from fecal samples of bats, 2 (two) harbored the *bla*TEM gene.

Conclusion: The discovery of the *bla*TEM gene in bat fecal samples indicates the potential for wild animals, especially bats, to spread ESBL resistance genes to the environment and to humans.

Keywords: *Escherichia coli,* Multidrug resistance*,* Bats, *bla*TEM, Human health.

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Introduction

Escherichia coli is a typical intestinal flora found in both humans and animals (Widodo *et al*., 2022). Some strains of *E. coli* are pathogenic, which can cause gastroenteritis, cystitis, pneumonia, and septicemia (Dutta *et al*., 2017). *Escherichia coli* is generally used as an indicator of antibiotic resistance because it can easily transfer antibiotic-resistance genes to other bacterial strains in the Enterobacteriaceae family, especially through plasmid mediation (Radhouani *et al*., 2012). Feces have been identified as a major reservoir of *E. coli* that harbor extended-spectrum beta-lactamase (ESBL) genes such as bla_{CTX-M}, bla_{TEM}, and bla_{SHV} (ElBaradei *et al*., 2020). Numerous antibiotic-resistant Enterobacteriaceae bacteria have been isolated from wild animals in recent years (Benavides *et al*., 2021; Benavides *et al*., 2022). The discovery of antibiotic-resistant Enterobacteriaceae bacteria in wild animals is a sign of the potential of wild animals as reservoirs (Dolejska and Literak, 2018). Activities such as human encroachment into wild animal habitats, transportation of wild animals, development of captive industries, and management of abused wild animals could be possible causes of antibiotic-resistant *E. coli* transmission from wild animals to humans (Rhyan and Spraker, 2010; Cunningham *et al*., 2017).

ESBLs display an extended substrate spectrum with the ability to hydrolyze a broader spectrum of antimicrobials belonging to the beta-lactam class that contains the oxyimino-group such as oxyimino-cephalosporins (e.g., ceftazidime and cefotaxime) as well as oxyimino-monobactam (aztreonam) (Guenther *et al*., 2011). Among microbes belonging to the Enterobacteriaceae family, the most common ESBLs are divided into four major families; TEM (Temoneira)-type beta-lactamases, CTX (cefotaximase)-M-type beta-lactamases, SHV (Sulfhydryl variable)-type beta-lactamases, and OXA (oxacillinase)-type beta-lactamases (Guenther *et al*., 2011). TEM-type beta-lactamases are well-known derivatives of TEM-1 and TEM-2. While the majority of TEM beta-lactamases are ESBLs, TEM-1, TEM-2, and TEM-13 are only able to hydrolyze penicillin derivates and thus are not regarded as ESBLs (Guenther *et al*., 2011). The *bla*TEM resistance gene to β-Lactam antibiotics in wild animals is an emerging phenomenon that is increasingly reported (Guenther *et al*., 2011). Since most wild animals are not given third-generation cephalosporin treatments, it is believed that contaminated food scraps, wastewater, domestic animal feces, and human contact are the main sources of ESBL-producing *E. coli* detected in wild animals (Homeier-Bachmann *et al*., 2022). Although there has not been much research on ESBL-producing *E. coli* in wildlife up to this point, it is concerning to think that wildlife may eventually serve as a reservoir for bacterial infections with the same pathogen that infects humans or other animals (Lagerstrom and Hadly, 2021). According to the latest data, ESBL-producing *E. coli* in wild animals is found to be widespread in several wild animal populations such as mammals, waterfowl, birds of prey, and rodents even though they are not exposed to antibiotics continuously (Atterby *et al*., 2017).

It is estimated that there are 230 bat species in Indonesia or around 21% of the bat species in the world (Fajri *et al*., 2014). Of these species, 77 species are grouped into the Megachiroptera suborder while 153 species are grouped into the Microchiroptera suborder (Fajri *et al*., 2014; Allocati *et al*., 2016). Lombok Island is an island that has quite a high diversity of bat species (Fajri *et al*., 2014). Bats are natural reservoir hosts and a source of infection for several microorganisms and have the potential to become vectors for the spread of zoonotic diseases (Letko *et al*., 2020). Several bacteria such as *Salmonella* spp., *Pasturella* spp., *E. coli*, *Leptospira* sp., and *Bartonella* spp. have been isolated from bats in various countries around the world (Allocati *et al*., 2016).

Caves are well-known roosting places for several types of bats; however, they have also been found in residential areas (Maulany *et al*., 2019). Based on a research survey conducted by Fajri *et al*. (2014), there is a cave in Lombok that has various bat species, namely the Tanjung Ringgit Giant Cave. Based on the above background, it is necessary to conduct a study on the detection and identification of ESBL-producing *E. coli* in bats residing in caves on Lombok Island. The results are expected to illustrate the potential of bats as reservoirs for the spread of ESBL-producing *E. coli* to the community.

Materials and Methods

Sample collection

The research was carried out from March 2023 to June 2023. A total of 150 bat fecal swab samples were aseptically collected in Tanjung Ringgit cave, Lombok Island, Indonesia, and immediately transported within 1–2 hours in ice packs to the laboratory for bacteriological analysis.

Isolation and identification of E. coli

All bat fecal swab samples were streaked onto eosin methylene blue agar (EMBA) medium and incubated for 18–24 hours at 37ºC. After incubation, colonies typical of *E. coli* (green metallic sheen) were further subjected to other physiological and biochemical characterization such as Gram staining, indole, methyl red, and Voges-Proskauer tests.

Antibiotic sensitivity test

Antimicrobial susceptibility testing was done by disc diffusion method on Mueller-Hinton agar according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2017). A suspension was prepared from a 24-hour growth of the test organisms in sterile water to match the 0.5 McFarland turbidity standard. This was seeded on the entire surface of the solidified Mueller-Hinton (MH) agar plate. The following antimicrobials (Oxoid, UK) were tested against the isolates: amoxicillin (10 µg), trimethoprim-

sulfamethoxazole (23.75 µg), ceftazidime (30 µg), streptomycin (10 µg), and tetracycline (30 µg). The inoculated MH agar plates were then incubated at 37°C for 18–24 hours. Inhibition zone diameters were measured, recorded, and interpreted as resistant or susceptible according to the CLSI guidelines (CLSI, 2017). Isolates with intermediate resistance were classified as "resistant" in this study.

blaTEM gene detection

The primer sequences used in this study were primers *bla*TEM-*Forward* 5'-ATGAGTATTCAACATTTCCG-3' and *bla*TEM-*Reverse* 5'-CTGACAGTTACCAATGCTTA-3' (Ballhausen *et al*., 2014). The marker used in this PCR method is Invitrogen 867 bp.

The initial stage of PCR amplification begins with making a reaction mixture which is carried out in cold conditions. The reaction mixture contained 12.5 µl of Go tag green master mix, 1 µl of *bla*TEM gene forward and reverse primers each, 0.5 µl of DNase-free water and 5 µl of DNA template (Ballhausen *et al*., 2014). A total of 20 µl of reaction mixture was put into Eppendorf PCR tubes, then amplified using a thermal cycler machine according to the protocol carried out by Fouladi *et al*. (2011) with slight modifications, namely predenaturation at 95°C for 1 minute followed by 40 denaturation cycles at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The amplification stage ended with a final extension at 72°C for 2 minutes. Electrophoresis was run in a 2% agarose gel with SYBR® safe DNA gel stain at 100 V and 250 mA for 35 minutes. The amplified PCR products were visualized in a UV transilluminator.

Ethical approval

Animal ethics approval was obtained via the ethical clearance committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia (Ethics no: 1.KEH.046.03.2023).

Result

Isolation of E. coli bacteria

Pure colonies of recovered isolates had a green metallic sheen appearance on the EMBA medium (Fig. 1). Physiological and biochemical test results indicated that the isolates were Gram-negative rods (coccobacilli), and were positive for motility, indole, and methyl red tests but negative for Voges-Proskauer and citrate utilization tests.

Out of the 150 bat fecal samples obtained from Tanjung Ringgit cave, Lombok Island, Indonesia, 56 (37%) were positive for *E. coli*.

Antimicrobial susceptibility testing results

The *E. coli* isolates recovered in this study showed resistance to amoxicillin, tetracycline, trimethoprim-sulfamethoxazole, streptomycin, and ceftazidime (Fig. 2, Tables 1 and 2). Eight (14.28%) isolates were

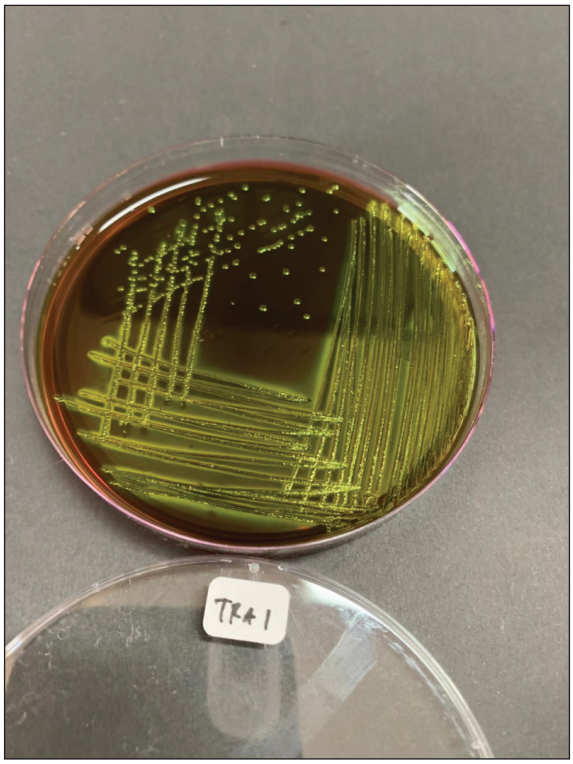

Fig. 1. Metallic green colonies of *E. coli* isolates on EMBA medium.

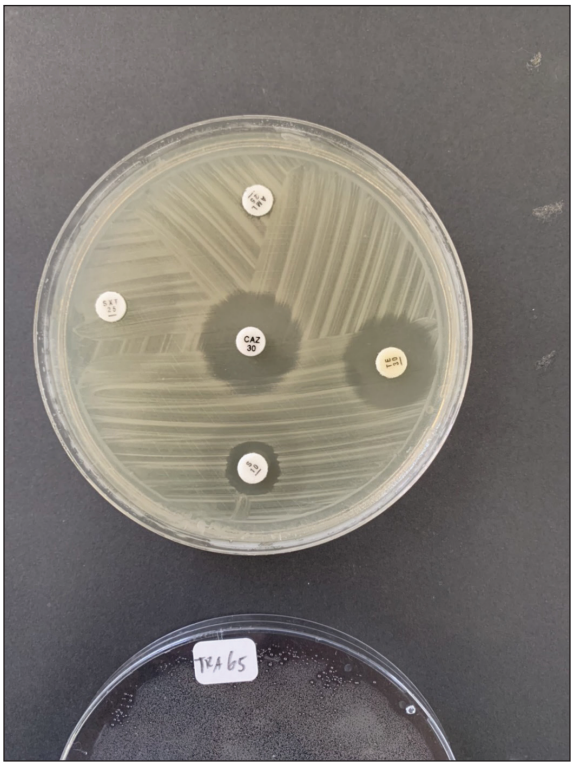

Fig. 2. Antibiotic sensitivity test results showing an antibiotic-resistant *E. coli* isolate.

Table 1. Antimicrobial susceptibility profiles of the recovered *E. coli* isolates.

Group of antibiotics	Resistance profile	Number of isolates (n = 71) Resistant isolates (%)	Total number of isolates (%)
0	No one is resistant	32 (57.14%)	32 (57.14%)
1	TE	2 (3.57%)	10 (17.85%)
	AML	8 (14.28%)	
2	AML—S	2 (3.57%)	6 (10.71%)
	TE—S	1 (1.78%)	
	S—CAZ	3 (5.35%)	
≥3	AML—TE—TS	1 (1.78%)	8 (14.28%)
	AML—TE—S	1 (1.78%)	
	AML—S—CAZ	4 (7.14%)	
	AML—TS—CAZ	1 (1.78%)	
	AML—S—TS—CAZ	1 (1.78%)	

(S): Streptomicin; (CAZ): Ceftazidime; (TS): Trimethoprim-sulfamethoxazole; (TE): Tetrasiklin; (AML): Amoxicilin.

Table 2. Antibiotypes of the recovered multidrug-resistant *E. coli* isolates.

Sample code	Resistance profile	Antibiotic				
		AML	TE	S	TS	CAZ
TRA 64	AML—TE—TS	✓	✓	–	✓	–
TRA 65	AML—S—TS—CAZ	✓	–	✓	✓	✓
TRA 69	AML—S—CAZ	✓	–	✓	–	✓
TRA 98	AML—TS—CAZ	✓	–	–	✓	✓
TRA 113	AML—S—CAZ	✓	–	✓	–	✓
TRA 120	AML—S—CAZ	✓	–	✓	–	✓
TRA 129	AML—S—CAZ	✓	–	✓	–	✓
TRA 133	AML—TE—S	✓	✓	✓	–	–

(✓): Resistant; (S): Streptomicin; (CAZ): Ceftazidime; (TS): Trimethoprim-sulfamethoxazole; (TE): Tetrasiklin; (AML): Amoxicilin.

observed to be multidrug-resistant as they exhibited resistance to at least 3 (≥3) different classes of antibiotics (Tables 1 and 2).

Detection of the E. coli encoding blaTEM ESBL gene

All 8 multidrug resistance (MDR) *E. coli* isolates recovered in this study were screened for the presence of *bla*TEM ESBL gene by PCR. Out of the 8 MDR *E. coli* screened, two (TRA 64 and TRA 98) harbored *bla*TEM ESBL gene (Fig. 3).

Discussion

In this investigation, we identified antibiotic-resistant *E. coli* in bat fecal samples from Tanjung Ringgit cave, Lombok Island, Indonesia. The recovered *E. coli* isolates showed resistance to amoxicillin, tetracycline, trimethoprim-sulfamethoxazole, streptomycin, and ceftazidime. Eight (14.28%) isolates were noted to exhibit multidrug-resistant traits (resistance to at least three classes of antibiotics) with two of them harboring the *bla*TEM gene.

The resistance profiles found in *Salmonella* spp., *Staphylococcus* spp., and *E. coli* in bat feces which contaminate water have been found to increase the transmission of resistant and pathogenic bacteria between wildlife, livestock, and humans (Uddin *et al*., 2020).

Escherichia coli is a bacterium that has the potential to harbor resistance genes to various antibiotics (Anshariet*a et al*., 2021; Yanestria *et al.,* 2022). The transmission of resistance genes to other bacteria in the vicinity is facilitated by bacterial plasmids, which are identified as the source of antibiotic resistance (Promite *et al*., 2017). *E. coli* can act as a reservoir for the spread of multidrug resistance to humans and the environment (Promite *et al*., 2017).

Wibisono *et al*. (2020) reported a relatively high resistance frequency to aztreonam (86.36%),

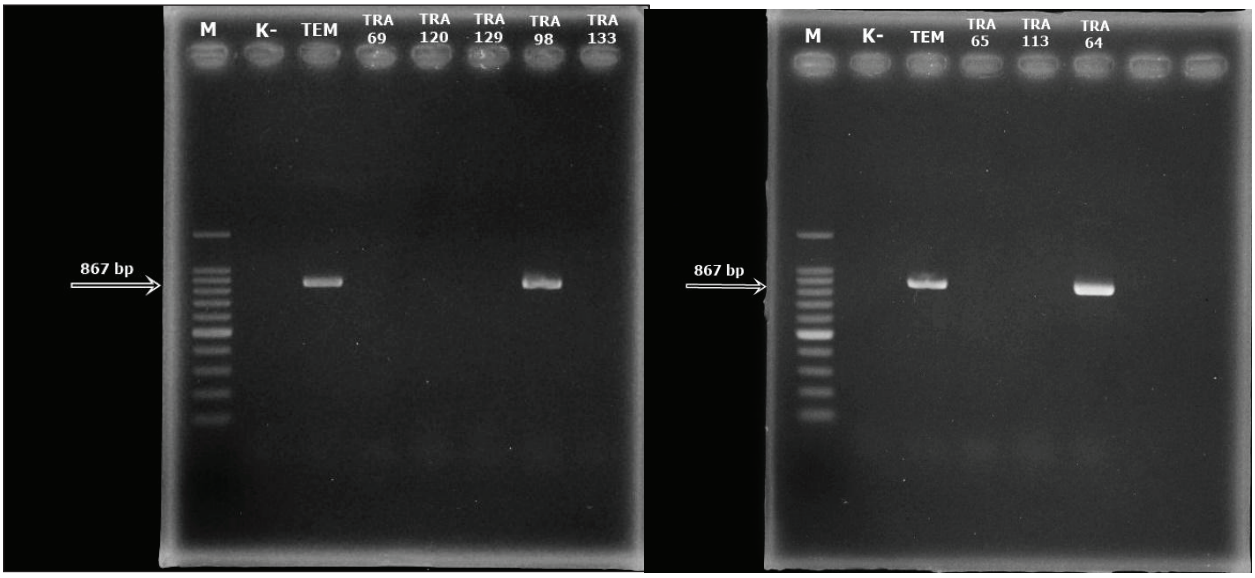

Fig. 3. The results of the detection of the *bla*TEM ESBL-encoding gene in MDR *E. coli* recovered from bat feces taken from Tanjung Ringgit Cave, East Lombok.

trimethoprim-sulfamethoxazole (77.27%), gentamicin (72.73%), and ciprofloxacin (68.18%) among ESBL-producing bacterial pathogens.

There have been reports of multidrug-resistant Enterobacteriaceae in wildlife all over the world (Kabali *et al*., 2021; Lagerstrom and Hadly, 2021; Homeier-Bachmann *et al*., 2022), but it is still unclear how wildlife contributes to the spread of antibiotic resistance. ESBL-producing *E. coli* isolated from bats was found to have a fairly high prevalence of antimicrobial resistance (Benavides *et al*., 2022). MDR *E. coli* has been reported to be found in fruit bats in Africa, Portugal, and Nigeria. MDR *E. coli* was found to be resistant to beta-lactamase, carbapenem, and fluroquinolone antibiotics (McDougall *et al*., 2022). Resistance found in wild animals may be acquired through direct contact with livestock, humans, or the environment (Kabali *et al*., 2021).

The level of tetracycline resistance is quite high due to its common use in the veterinary field, followed by the use of other classes of antibiotics used in this study, namely aminoglycosides such as streptomycin, and sulfonamides such as trimetroprim-sulfamethoxazole (Hunter *et al*., 2010). The tetracyclines, aminoglycosides, and the combination drug trimetroprim-sulfamethoxozole have a significant impact on microbial activity in the digestive tract; so, they are often used to treat gastrointestinal diseases (Ramandinianto *et al*., 2020; Kakoullis *et al*., 2021).

To the best of our knowledge, and for the first time in Indonesia, we isolated *E. coli* harboring *bla*TEM ESBL gene in bat feces from Lombok Island Cave. Research conducted by Effendi *et al*. (2022) found that MDR *E. coli* bacteria isolated from pigs harbored 15.6% of the *bla*TEM gene.

A beta-lactamase enzyme known as *bla*TEM is frequently found in ESBL-producing bacterial isolates, especially *E. coli* strains. Beta-lactamase family members of TEM, SHV, and CTX-M are also frequently found in other Enterobacteriaceae bacteria (Guenther *et al*., 2011). Research conducted on *E. coli* isolates that were positive for the DDST test found the *bla*TEM gene and the *bla*CTX-M gene using multiplex PCR (Ansharieta *et al*., 2021). This is proven by research conducted by Benavides *et al*. (2022) detecting eight β-lactamase genes including the *bla*TEM, *bla*OXA, and *bla*CTX-M genes from bat feces samples. In another previous study, Benavides *et al*. (2018) also found the same ESBL-encoding gene, *bla*CTX-M-15, in the plasmid of *E. coli* bacteria isolated from pig and bat feces, indicating cross-contamination of ESBL-producing bacteria between bats and livestock.

The spread of ESBL-producing Enterobacteriaceae bacteria is one of the public health problems in the world today (Effendi *et al*., 2021) as it could complicate treatment options and further result in serious economic burden and losses. Enzymes generated by bacteria harboring ESBL genes can hydrolyze beta-lactam antibiotics, including third and fourth-generation cephalosporins (Riwu *et al.,* 2020; Faridah *et al.,* 2023). Several ESBL variants, such as *bla*TEM, blaSHV, and blaCTX-M, are currently found on bacterial plasmids (Benavides *et al*., 2021; Wibisono *et al.,* 2021).

In general, wild animals do not come into direct contact with antibiotics; wild animals may be exposed to antibiotic resistance through food and drink consumed in contaminated environments (Savin *et al*., 2020). International organizations such as the Food and Agriculture Organization are also starting to pay attention to the interconnectedness of wildlife,

humans, and livestock regarding antibiotic resistance (Wall *et al*., 2016). The increasing interaction between humans, livestock, and wild animals can increase the potential for transmission of ESBL-producing *E. coli* (Kabali *et al*., 2015). Thus, the detection of antibiotic resistance, especially ESBL-producing *E. coli* in nature and wild animals, is a concern and requires further research.

Conclusion

In total, from 150 bat feces samples isolated, 8 samples were positive for MDR *E. coli*. Eight (14.28%) *E. coli* isolates were noted to exhibit multidrug-resistant traits with two of them harboring the *bla*TEM gene. This indicates the potential for wild animals, especially bats, to spread ESBL resistance genes to the environment and humans.

Acknowledgment

The authors thank the Faculty of Veterinary Medicine, Universitas Airlangga.

Conflict of interest

The authors declare that there is no conflict of interest.

Funding

This study was supported in part by the Penelitian Unggulan Airlangga (PUA) Universitas Airlangga, Indonesia, in the fiscal year 2023, with grant number: 1710/UN3.LPPM/PT.01.03/2023.

Data availability

All data supporting the findings of this study are available within the manuscript and no additional data sources are required.

Author's contributions

Conceptualization and design: YRM and KNK; acquisition of data: AH and IBM; formal analysis and interpretation of data: OSMS and MEES; writing-original draft preparation: ARK and SCK; writing-review and editing: ALDA, YP, and MHE. All authors have read and agreed to the published version of the manuscript.

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