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Cave Bats as Carriers of Extended Spectrum Beta-lactamase Produced by *Escherichia coli* from the Island of Lombok, Indonesia

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ABSTRACT

Background: *Escherichia coli* is a Gram-negative commensal bacterium that normally resides in the digestive tract of humans and animals which is used as an indicator of the extended-spectrum beta-lactamase (ESBL) gene in an individual and can carry out gene transfer. Transmission of ESBL *E. coli* from wild animals has not been given much attention even though wild animals can act as vectors that spread ESBL in the environment so that it can pose a risk to public health. This research aims to identify the blaTEM gene which codes for ESBL in *E. coli* from bats that live in caves in Lombok, Indonesia.

Methods: A total of 135 samples from bat rectal swabs were cultured on Eosin Methylene Blue Agar media and biochemical suspects were identified using the Indole, Methyl red, Voges-Proskauer, Citrate + and H_2S (IMVIC) test. The *E. coli* bacteria obtained were tested for sensitivity using 7 classes of antibiotics, namely amoxicillin, ciprofloxacine, sulfamethoxazole/trimethoprim, tetracycline, gentamicin, cefotaxime and azithromycin. Bacteria that show multidrug resistance are subjected to PCR testing to detect the blaTEM gene.

Result: Of the 135 test samples, it was found that 97 (71.85%) samples were positive for *E. coli*, 12 (12.37%) samples were Multidrug Resistance (MDR) and 2 (2.06%) samples had the blaTEM gene as the ESBL coding gene in bats that live in caves on the island of Lombok, Indonesia. The presence of the blaTEM gene in *E. coli* from bats can be indicated as a reservoir for MDR and ESBL transmission so that it can have an impact on public health.

Key words: Bats, blaTEM, *E. coli*, ESBL, Public health.

INTRODUCTION

Escherichia coli is a Gram-negative commensal bacterium that normally resides in the digestive tract of humans and animals which is used as an indicator of the ESBL gene in an individual and can transvert the gene in other bacterial species (Effendi *et al.,* 2022). Extended spectrum beta-lactamase (ESBL) is an enzyme produced by Enterobacteriaceae which has experienced antimicrobial resistance (AMR) which can hydrolyze third generation cephalosporins including cefotaxime, ceftazidim ceftriaxone and monobactam (Ansharieta *et al.,* 2021). The blaTEM, blaSHV, blaCTX-M and blaOXA genes are the types that often appear to encode ESBL (Wardhana *et al.,* 2021). The blaTEM and blaSHV genes only have a few differences in amino acid substitutions that have unique characteristics carried by transposons, which makes it difficult to treat Enterobacteriaceae ESBL infections (Stone *et al.,* 2023).

The presence of the ESBL gene in wild animals were contamination bacterial was contain ESBL gen from humans and animals in environmental pollution, wastewater treatment plants, food, agriculture, fisheries (fertilizer from feces, water, hospital waste and raw vegetables) (Rojas-Sereno *et al.,* 2023; Loayza *et al.,* 2020). The incidence of antibiotic resistance of *E. coli* in wildlife in Indonesia has been reported in land mammals (Lagerstrom *et al.,* 2021; Nisa *et al.,* 2021), birds (Huy *et al.,* 2021; Umar *et al.,* 2018) and bats (Mustika *et al.,* 2024). Test from 115 samples swab

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broiler chicken in Indonesia showed that 33, 04% was multi-drug resistance (MDR) and from 65 wastewater samples showed that 36,92% resistance to antibiotics amoxicillin, ampicillin, sulbactam, cefazolin, cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam, gentamicin, ciprofloxacin and trimethoprim/sulfamethoxazole (Yanestria *et al.,* 2022). The *Escherichia coli* isolated from chicken in wet markets showed that was resistant to antibiotics aztreonam, chloramphenicol and gentamicin (Effendi *et al.,* 2021a). CTX-M-15 is the enzyme most widely distributed as the cause of ESBL-producing *E. coli*, while the aac resistance gene conferring resistance to aminoglycosides (tobramycin and amikacin) and ciprofloxacin is often detected in association with CTX-M-15 (Banerjee *et al.,* 2013). In line with research conducted by Wibisono *et al.* (2021), 80% (8/10) isolated MDR have gen CTX-M. Gen ESBL produced from *E. coli* is useful for understanding pathogenesis and public health (Effendi *et al.,* 2022; Zahra *et al.,* 2024). Apart from CTX-M, blaTEM is a gene found as an ESBL *E. coli* in bats (Graces *et al.,* 2019).

It is estimated that there are 230 bat species in Indonesia or around 21% of the bat species in the world. Of these species, 77 species are grouped into the Megachiroptera suborder and 153 species are grouped into the Microchiroptera suborder. Lombok Island is an island that has quite a high diversity of bat species (Mustika *et al.,* 2024). 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 (Allocati *et al.,* 2016). Several bacteria such as *Salmonella* spp., *Pasturella* spp., *E. coli*, *Leptospira* sp. and *Bartonella* spp. has been isolated from bats in various countries around the world (Allocati *et al.,* 2013).

Bats are generally found in caves and residential areas (Garces *et al.,* 2019). Caves are a roosting place for several types of bats (Az-Zahra *et al.,* 2023). The presence of bats in caves can act as a key provider of ecosystem energy for organisms in the cave (Medellin *et al.,* 2017). A cave is a space formed by water-dissolving activity and has spatial divisions based on light intensity. The characteristic of caves is the diversity of habitats within them (Wasti *et al.,* 2021). Based on a research survey conducted by Mustika *et al.* (2024) there are two caves in Lombok that have various bat species, namely Lawah Cave and Saung Pengembur Cave. These two caves are caves that are always visited by foreign and domestic tourists.

The proximity of humans and wild animals is often associated with cross-contamination and transmission of ESBL-producing *E. coli* because transmission from wild animals is often underestimated, even though wild animals are definitely a vector of resistance (Nicolas-Chanoine *et al.,* 2014). The aim of this research is to determine the level of susceptibility to ESBL-producing *E. coli* in wild bats on the island of Lombok, Indonesia. It is hoped that the research results will illustrate the potential of bats as a reservoir for the spread of ESBL-producing *E. coli* on public health.

MATERIALS AND METHODS

Ethical approval

The ethical clearance committee of the Faculty of Veterinary Medicine at Universitas Airlangga, Indonesia provided consent for the use of animals (No. 1.KEH.046.03.2023).

Study area and sample collection

The research was conducted from September 2023 to November 2023. Samples were obtained by swabbing the rectum of live bats caught from caves on the island of Lombok, namely Gua Lawah in West Lombok and Saung Pengembur cave in Central Lombok. Bats were caught using mist nets 20 × 20 mm mesh from 4 pm to 10 pm (McDougall *et al.,* 2021). The nets were always monitored and if any bats were caught in the net they were taken periodically and placed in the cage. Bats caught were handled for rectal swabs. The sampling in this research was approved by the West Nusa Tenggara Natural Resources and Conservation Agency.

Sample collection was carried out by cleaning the hairs around the anus using 70% isopropyl alcohol to avoid bacterial contamination from the hair, A sterile cotton swab was inserted into the rectum and rotated for a few moments to ensure that bacteria adhered to the cotton swab (Adesiyun *et al.,* 2009). The swab were placed in transport media (Oxoid, UK) to be taken to the laboratory.

Isolation and identification of *E. coli*

Using Eosin Methylene Blue Agar (EMBA) medium (HI MEDIA M317), *E. coli* bacteria were isolated by inoculating sample on media and then incubating for 18 to 24 hours at 37°C. Gram staining and the biochemical assays. Indole, Methyl red, Voges-proskauer, citrate (IMVIC) and H_2S were used to identify *E. coli* (Dameanti *et al.,* 2023; Debbarma *et al.,* 2023). Bacterial isolation and sensitivity test were done at the laboratory of bacteriology, Faculty of Veterinary Medicine, Airlangga University.

Antibiotic sensitivity test

The sensitivity test used the disk diffusion method (diffusion test) on Mueller-Hinton Agar (MHA) media (HiMedia M173)

Table 1: Primer arrangement used in the PCR method.

Gene	Primer	Base pair	Reference
blaTEM	F: 5′-ATGAGTATTCAACATTTCCG-3′ R: 5′-CTGACAGTTACCAATGCTTA-3′	867 bp	(Naelasari *et al.,* 2018)

Note: F= Forward; R= Reverse.

adapted to the Clinical Laboratory Standards Institute (CLSI) 2020. The antibiotic disk (Oxoid, England) used in this study belonged to seven classes, including amoxicillin 25 µg (AML) (Oxoid, UK), ciprofloxacine 5 µg (CIP) (Oxoid, UK), sulfamethoxazole/trimethoprim 25 µg (SXT) (Oxoid, UK), tetracycline 30 µg (TE) (Oxoid, UK), gentamicin 10 µg (CN) (Oxoid, UK), cefotaxime 30 µg (CTX) (Oxoid, UK), azithromycin 15 µg (AZM) (Oxoid, UK). One to two colonies were taken from EMBA media using a sterile tube, then placed in physiological NaCl and opacity adjusted to Mc Farland standard 0.5 (1.5 × 108 CFU/ml). A loopful of bacterial suspension was smeared on the mueller hinton agar (MHA) media all over with a sterile swab. Incubated the plate in an aerobic environment for 16-18 hours at 35°C and interpretation of the findings was done using CLSI 2020 to modify the inhibitory zone's diameter (Foti *et al.,* 2023; Debbarma *et al.,* 2024). When bacteria in an isolate were resistant to three or more distinct antibiotic classes, the isolate was deemed Multidrug Resistance (MDR). Presumptive ESBL is known if there are bacterial resistance to ≥3 types of antibiotics from different groups (Wibisono *et al.,* 2020).

blaTEM gene detection

Presumptive ESBL strains were then continued with molecular screening for the blaTEM gene by DNA extraction using QIAamp® DNA kit (QIAGEN, Germany). blaTEM primers used in this study can be seen in Table 1. Thermal cycle pre-denaturation temperature was 96°C for 5 minutes followed by denaturation for 1 minute at 96°C, 35 cycles of annealing at 58°C for 1 minute and extension at 72°C for 1 minute. Amplification ended with final extension at 72°C for 10 minutes. After that, the amplicons were visualized by electrophoresis using 2% agarose gel. PCR test was taken in gastrointestinal laboratoty, Institute of Tropical Disease, Airlangga University.

RESULTS AND DISCUSSION

The result of isolation and identification of *Escherichia coli* showed that 97 (71.85%) were positive *E. coli* from 135 sampeles of rectum swabs based on morphological culture, Gram staining and biochemical tests (Table 2). *E. coli* produce metallic green bacterial colonies on EMBA media (Fig 1). Gram staining revealed the presence of Gram negative short rods. The IMViC test revealed bacterial strains to be positive for indole, MR and motility, but negative for indole and citrate utilization tests, motile MR positive thus confirming isolates to be *E.coli* by biochemical tests

Based on the outcomes of the antibiotic resistance test, the antibiogram revealed that, out of the 97 *E. coli* isolates, 20 (20.62%) were resistant to one class of antibiotics and 24 (24.74%) resistant to two classes. Twelve isolates (12.37%) that exhibited resistance to three or four different antibiotic classes were identified as MDR (Table 3 and Fig 2). The dominant antibiotic resistance pattern was CIP - AML - SXT - AZM - TE with 6 isolates (Table 4). Following the detection of the blaTEM gene, it was determined that 12 *E. coli* isolates were multidrug resistant based on the findings of the sensitivity test. Two *E. coli* isolates tested positive for blaTEM based on the results of the electrophoresis. The two isolates are with sample codes 2.31 and 2.70 (Fig 3).

Research into antibiotic resistance in wild animals has recently begun to be carried out because the uncontrolled movement of these wild animals can have serious impacts on public health. AMR research in wild animals, such as bats, can provide an overview of the exposure received by wild animals and the potential for spread of ESBL from wildlife to the environment. Bats have the ability to fly an average distance of 26.14 km (0.33-97.57 m) (Randhawa *et al.,* 2020). The small amount of research on wild animals can make the picture of the spread of ESBL in the environment still not very clear, there are only a few studies on MDR from bats in Indonesia, including research conducted by Masrukhin *et al.* (2021) on bat isolates in Gunung Halimun Salak National Park.

Multidrug resistance to antibiotics in Gram-negative bacteria resulting from treatment or exposure to antibiotics in the environment can be found in *E. coli*, *Enterobacter* spp., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinebacter baumannii* (Ramatla *et al.,* 2024; Riwu *et al.* 2022). The macrolide antibiotic Azithromycin is an antibiotic that often appeared as a cause of resistance in this study. Beta-lactam and macrolide antibiotics are antibiotics that are often used to treat bacterial respiratory tract infections in humans (Ríos *et al.,* 2024). In Japan, from 82 nasal swab samples from human patients, it was found that 23 *Klebsiella pneumonia* bacteria and *E. coli* had the ESBL gene, 15 *E. coli* isolates from 44 patients were known to produce ESBL, where the proportion of administration using carbapenem, cephem and macrolide antibiotics was very significant (Matsumoto *et al.,* 2023). From food product etawa milk in Indonesia found that 100% of samples was DDST test were found blaTEM gene (Tyasningsih *et al.,*

Table 2: Results of isolation and identification of *E. coli.*

Location	Sample size	Identification test							Positive *E. coli* (%)
		EMBA	Gram stain	IMViC test					
				Indol	Motile	MR	VP	Citrate	
Lawah cave	50	50	45	34	34	34	34	34	34 (68)
Saung Pengembur cave	85	85	77	63	63	63	63	63	63 (74.12)
Total	135	135	112	97	97	97	97	97	97 (71.85)

Note: % (Positive percentage).

2022). mefB is an ARGs of *E. coli* in poultry in kwara state, north central nigeria which mutates to cause resistance to macrolide antibiotics (erythromycin, azithromycin and tylosin) (Al-Mustapha *et al.,* 2023). The antibiotic azithromycin is a macrolide antibiotic which is widely used for therapy in humans and livestock. The macrolide antibiotic functions to inhibit bacterial proteins between gram-negative and gram-positive bacteria. This antibiotic is used for the treatment of respiratory and digestive, genital and skin infections (Monahan *et al.,* 2023).

Fig 1: *E. coli* bacteria purified after biochemical tests.

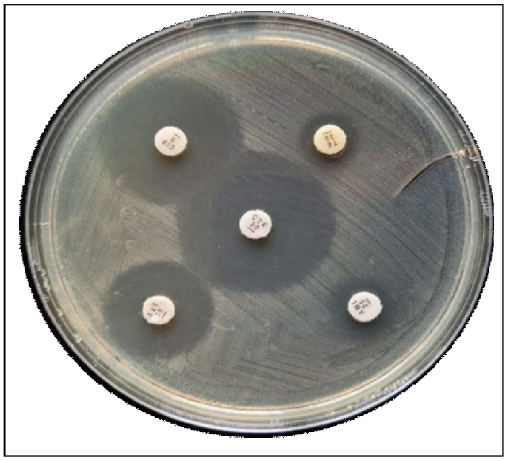

Fig 2: Sensitivity test of *E. coli* bacteria isolates.

Of the 97 *E. coli* isolates, 25 samples were found to be resistant to the antibiotic amoxicillin. Amoxicillin is a beta-lactam antibiotic that is used to treat pathogenic bacteria by inhibiting cell wall synthesis. Amoxicillin is often used for treatment because it is more easily absorbed than other antibiotics (Zhang *et al.,* 2023). The high incidence of AMR on cattle farms can occur in cows that have not yet been weaned, because the immune system is not yet fully formed and antibodies from colostrum have begun to decline so the risk of infection becomes very high (Merle *et al.,* 2023). Research conducted by Ningtyas *et al.* (2024) on water sources in chicken farms in West Lombok showed that the isolated *E. coli* bacteria were resistant to penicillin (100%) and tetracycline (75%) antibiotics, the researchers believe that the bacteria that have developed resistance come from chickens on the farm. The similarity of antibiotic resistance of *E. coli* bacteria in humans, animals and the environment show that the closeness between humans, animals and the environment makes the circulation of antibiotic resistance unavoidable (Barker *et al.,* 2023).

All samples in this study showed susceptibility to the antibiotics cefotaxime (100%) and gentamicin (98.97%). The antibiotic gentamicin is an aminoglycoside antibiotic which has recently been used to treat Gram-negative and Gram-positive bacterial infections in the field of veterinary medicine. Amoxicillin and cefotaxime are indicators of resistance to the beta-lactam class of antibiotics which can carry the ESBL gene (Paramitadevi *et al.,* 2024). In contrast to the results of research on isolates from bats in Peru, more than 60% of the isolates were resistant to second and third generation penicillin and cephalosporin

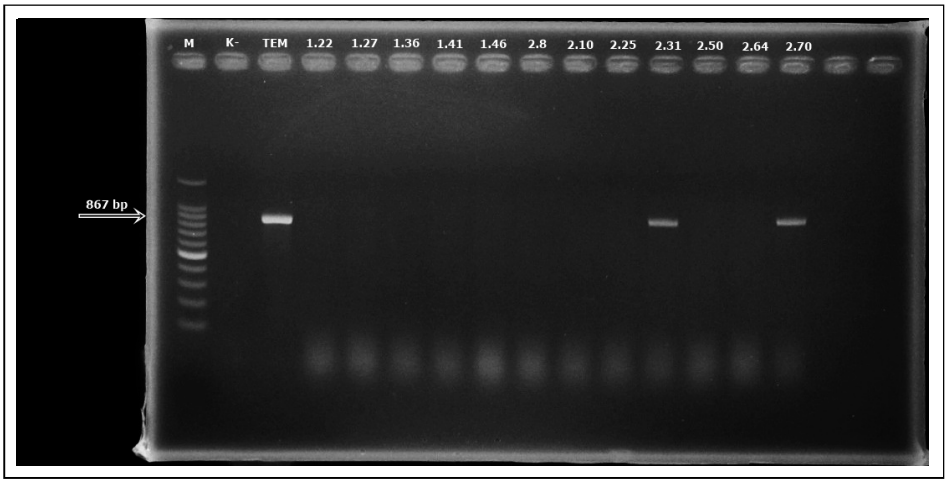

Fig 3: Detection of the blaTEM gene encoding ESBL in *E. coli* bacteria from bat rectal swabs.

Table 3: Zone of inhibition in the sensitivity test using the disk diffusion test method.

Group of antibiotics	Resistance profile	Number of isolates (n=97) Resistant isolates (%)	Total number of isolates (%)
0	No one is resistant	41 (42.27)	41 (42.27)
1	AML	5 (5.15)	20 (20.62)
	SXT	3 (3.09)	
	AZM	10 (10.31)	
	TE	1 (1.03)	
	CN	1 (1.03)	
2	CIP-AML	1 (1.03)	24 (24.74)
	CIP-SXT	1 (1.03)	
	AZM-CIP	1 (1.03)	
	CIP-TE	1 (1.03)	
	AML-SXT	2 (2.06)	
	AML-AZM	6 (6.18)	
	SXT-AZM	1 (1.03)	
	SXT-TE	4 (4.12)	
	TE-AZM	7 (7.22)	
≥3	CIP-SXT-AZM-TE	1 (1.03)	12 (12.37)
	CIP-AML-SXT-TE	1 (1.03)	
	AML-SXT-AZM	1 (1.03)	
	AML-SXT-AZM-TE	3 (3.09)	
	CIP-AML-SXT-AZM-TE	6 (6.18)	

Note: AML= Amoxicillin, TE= Tetracycline, SXT= Sulfamethoxazole/trimethoprim, CIP= Ciprofloxacine, AZM= Azithromycin, CN= Gentamicin.

Table 4: Results of identification of multidrug resistant *E. coli* bacteria.

Sample code	Resistance profile	Antibiotic				
		CIP	AML	SXT	AZM	TE
1.22	AML-SXT-AZM	–	✓	✓	✓	–
1.27	CIP-AML-SXT-AZM-TE	✓	✓	✓	✓	✓
1.36	CIP-AML-SXT -AZM-TE	✓	✓	✓	✓	✓
1.41	CIP-AML-SXT-AZM-TE	✓	✓	✓	✓	✓
1.46	AML-SXT-AZM-TE	–	✓	✓	✓	✓
2.8	CIP-AML-SXT-AZM-TE	✓	✓	✓	✓	✓
2.10	AML-SXT-AZM-TE	–	✓	✓	✓	✓
2.25	CIP-AML-SXT-AZM-TE	✓	✓	✓	✓	✓
2.31	AML-SXT-AZM-TE	–	✓	✓	✓	✓
2.50	CIP-SXT-AZM-TE	✓	–	✓	✓	✓
2.64	CIP-AML-SXT-AZM-TE	✓	✓	✓	✓	✓
2.70	CIP-AML-SXT-TE	✓	✓	✓	–	✓

Note:✓= Resistant, CIP= Ciprofloxacine, AML= Amoxicillin, SXT= Sulfamethoxazole/trimethoprim, AZM= Azithromycin, TE= Tetracycline.

antibiotics (Benavides *et al.,* 2022). The discovery of this pattern of antibiotic resistance is important for antibiotic management and the need for health service providers to choose the antibiotics that will be used for therapy as an effort to maximize treatment (Oboodi *et al.,* 2024; Farizqi *et al.,* 2023).

The discovery of *E. coli* bacteria in samples of bats that are MDR in this study and were detected to have the blaTEM gene is very risky for public health because bats are wild animals that can live anywhere and defecate any where so the risk of bat feces contamination of the environment is very large. Extended-spectrum beta-lactamase (ESBL) can be caused by the resistance of *E. coli* bacteria to beta-lactam antibiotics or bacterial plasmids that are resistant to aminoglycoside antibiotics, trimethoprim, quinolones, sulphonamides, chloramphenicol and tetracycline (Haddadin *et al.,* 2023). The Temoneria (TEM) and sulfhydryl variable (SHV) genes were genes that were very frequently found in patients in hospitals from 1980-1990, then cefotaxime (CTX-M) became dominant

due to the widespread use of third-generation cephalosporins (Nahar *et al.,* 2023). Of the 12 test samples that experienced multidrug resistance, two samples were found to be positive for the blaTEM gene. In line with research on bats in Nigeria, the blaTEM and multi-DHA genes were the predominant genes detected in resistant *E. coli* bacteria (Modupe *et al.,* 2022).

In Indonesia, the blaTEM gene is the dominant gene (70%) in ESBL *E. coli* in broiler chickens (Effendi *et al.,* 2021b), but it is different in layer chickens where blaCTX is the dominant gene that appears as the cause of ESBL followed by blaTEM and blaSHV (Wibisono *et al.,* 2020). The blaTEM gene confers resistance to early cephalosporin antibiotics and penicillins by excessively hydrolyzing the β-lactam ring and causing resistance to carbapenems and cephamycin (Ibrahim *et al.,* 2023). The blaTEM gene (83.8%) is the gene that often appears as the cause of ESBL in poultry in North Central Nigeria, samples show MDR to the antibiotics sulfamethoxazole, trimethoprim, tetracycline and ampicillin (Al-Mustapha *et al.,* 2023). The blaTEM-1B gene was 65% (n=13) the gene that appeared as the gene causing resistance to *E. coli* isolates from dogs in San Francisco and 70% (n=25) in dog owners, followed by blaTEM104 in one person without appearing in dogs lookout (Walas *et al.,* 2024).

The blaTEM gene can be found on DNA plasmids, the presence of the gene on the plasmid can facilitate gene transfer between different bacteria, other beta-lactamase genes are derivatives of blaTEM (Sah *et al.,* 2024). Data on antibiotic resistance and ESBL genes detected in this study can be used as reference data as literature for doctors and veterinarians as an illustration of AMR and ESBL in wild animals which can contaminate the human environment.

CONCLUSION

The level of antibiotic resistance in wildlife reflects the level of resistance in the environment, humans and animals. blaTEM is a gene detected from non-human *E. coli* isolates in Lombok, Indonesia. The one health approach is very important to implement for human, animal and environmental health as a step to prevent the spread of the discovered ESBL genes. Genetic characterization of ESBL genes can be used to identify potential zooanthroponotic and zoonotic risks that are beneficial to public health and bat health.

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Conflict of interest

All authors declare no conflict of interest.

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