Molecular detection of *Klebsiella pneumoniae* producing extended-spectrum beta-lactamase isolated from bat feces from the Tanjung Ringgit bat cave, Lombok Island, Indonesia

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**Abstract**

**Background and Aim:** Bats are a reservoir for the pathogenic bacteria *Klebsiella pneumoniae* and can spread it through feces that fall in nests/caves, carried, and dropped while they fly near human settlements, and from their saliva. The emergence and spread of multidrug resistance (MDR) strains of *K. pneumoniae* indicate that resistant to antibiotics, especially extended-spectrum beta-lactamase (ESBL), is considered an important global health threat. The aim of this study was to determine the presence of the gene encoding extended beta-lactamase in *K. pneumoniae* isolated from fresh bat feces collected from the Tanjung Ringgit bat cave, East Lombok.

**Materials and Methods:** In this study, 150 fresh fecal samples were analyzed using standard microbiological techniques for the presence of *K. pneumoniae*. *K. pneumoniae*-positive isolates were subjected to antibiotic sensitivity testing, followed by molecular detection using polymerase chain reaction.

**Results:** This study showed that 14 (9.3%) of 150 samples were positive for *K. pneumoniae*. Ten of the 14 samples (71.4%) were MDR isolates and 6 (42.9%) had the *blaSHV* gene identified.

**Conclusion:** The presence of *K. pneumoniae* isolated from fresh bat feces, which is MDR and has the *blaSHV* gene encoding ESBL indicates that bats can be a reservoir for the transmission of MDR and ESBL bacteria has an impact on public health in the study area.

**Keywords:** bat, *blaSHV*, extended-spectrum beta-lactamase, *Klebsiella pneumoniae*, public health.

**Introduction**

Bats belong to the large order Chiroptera and are widespread throughout the world, with many species that exploit urban and residential environments [1, 2]. The species is considered to be: Is a reservoir of many viruses that periodically spread in human populations during disease outbreaks and their dynamics. Bats are also known as potential reservoirs of pathogenic bacteria. Bats have a habit of looking for food and flying long distances, which can endanger the health of animals and humans in their cave/residence environments, especially those near the Tanjung Ringgit bat cave, East Lombok. Different types of viruses and bacteria have also been found in bats. Bats are reservoirs of pathogenic bacteria that *Klebsiella pneumoniae* can spread through feces that fall in nests/caves, are carried, and dropped from their saliva while they flew near human settlements [4]. Recently, *K. pneumoniae* has gained prominence as an infectious agent due to the increasing number of severe infections and an increasing scarcity of effective treatments [5]. Along *K. pneumoniae* is a major source of antibiotic resistance with its high prevalence. The World Health Organization (WHO) has also grouped *K. pneumoniae*...
as one of the important bacteria involved in antibiotic resistance and is the bacteria that most often spread its resistance genes [6]. Emergence and spread of multidrug resistance (MDR) strains of *K. pneumoniae* indicates resistant to antibiotics, particularly extended-spectrum beta-lactamase (ESBL) is considered an important global health threat [2]. Moreover, rapid spread of microorganisms with antimicrobial resistance (AMR) has increased isolation levels of MDR *K. pneumoniae* strains in humans. Some strains have different AMR activities gene patterns which have been isolated from several European countries [7]. According to the AMR monitoring system data, MDR *K. pneumoniae* in the population has dramatically increased recently [8]. Increasing reports of *K. pneumoniae* spreading in wild animals that are resistant to various types of antibiotics, including ESBL, also have important implications for the spread of ESBL genes and the environment. In addition, proximity to pets or animals, especially from bats, to the best of our knowledge, no studies have shown transmission between humans and wild animals or vice versa. To gain better insight into the origin of ESBLs from bats, antibiotic sensitivity testing and molecular analysis of *bla* genes from *K. pneumoniae* isolates will be inoculated on MHA media before extraction, which were then placed in a test tube containing 2% peptone water buffer and labeled after sampling; the sample was stored in a cooler in a standing position and was taken to the laboratory.

**Materials and Methods**

**Ethical approval**

Animal ethics approval was obtained from the Ethical Clearance Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia (Ethics no: 1.KEH.046.03.2023).

**Study period and location**

This study was conducted during March and April 2023. Samples were collected in Tanjung Ringgit Cave, East Lombok, West Nusa Tenggara, Indonesia. Sample isolation, antibiotic sensitivity test, and molecular detection was tested in a Veterinary Public Health Laboratory, Universitas Airlangga.

**Sample collection**

Sixty samples were collected in a bat cave in Tanjung Ringgit Regency, East Lombok. This cavern was chosen because it has a large population of bats of various species and is close to residential areas. A total of 150 fecal samples were collected and divided into two collections (75 samples were collected for each collection) with a sampling interval of 3 days. Each collection used a different fecal sample because it was taken from a different area of the cave. A sterile swab stick was used to collect fecal samples aseptically. The feces were then placed in a test tube containing 2% peptone water buffer and labeled after sampling; the sample was stored in a cooler in a standing position and was taken to the laboratory.

**Isolation and identification of *K. pneumoniae***

Samples of fresh bat feces were planted on MacConkey Agar (MCA; Oxoid, UK) media by streaking, which were incubated at 37°C for 18–24 h. Next, bacterial colonies were then observed growth on MCA media, including the shape, color, and edges of the colonies on the media. *K. pneumoniae* colonies on MCA media are typically mucoid pink [11]. Identify cell morphology using Gram staining. Subsequently, the isolate was continued with biochemical tests Sulfide Indole Motility test to test indole, sulfide, and bacterial movement. Methyl Red - Voges-Proskauer and citrate tests using Simmon’s citrate agar (Himedia, India).

**Antibiotic sensitivity test**

Antibiotic sensitivities were tested using the Kirby-Bauer agar diffusion method. Antibiotics that have been used are antibiotics packaged in disk form. The clear zone formed in this test was divided into three categories: sensitive, intermediate, and resistant. *K. pneumoniae* bacterial isolates were collected from 1 to 2 colonies using a tube and placed in physiological NaCl tested for turbidity using the McFarland 0.5 standard, 0.2 mL was taken and gently rubbed over the entire surface of Mueller–Hinton agar (MHA: Oxoid) media. Sensitivity testing was performed using five antibiotics (Oxoid): amoxicillin (AML) 10 μg, ceftazidime (CAZ) 10 μg, ciprofloxacin (CIP) 10 μg, Streptomycin (STR) 10 μg, and Trimethoprim-Sulfamethoxazole (TS) 10 μg. Media inoculated with *K. pneumoniae* were incubated at 37°C for 24 h.

**Detection of *bla*SHV gene in *K. pneumoniae* isolates**

*K. pneumoniae* culture obtained in MCA media was continued to detect the presence of the *bla*SHV ESBL gene using polymerase chain reaction (PCR) using primers *bla*SHV (Forward) and *bla*SHV (Reverse) (Table-1) [9]. *K. pneumoniae* isolates will be inoculated on MHA media before extraction, which was incubated at 37°C for 24 h. Several loops of...
bacterial colonies to be tested from one isolates were placed in Eppendorf safe-lock tubes containing 300 μL Tris EDTA (TE) (10 mM Tris, pH 8, 10 mM ethylenediaminetetraacetic acid). Subsequently, the suspension was vortexed and continued using the boiling lysis method by inserting the Eppendorf ThermoStat™ (Hamburg, Germany) at 98°C for 10 min. After that, it was centrifuged at 5000× g for 10 min and then stored at 20°C until PCR analysis. Subsequently, 20 μL of the reaction mixture (12.5 μL Go tag green master mix, 0.5 μL DNase-free water, and 5 μL DNA template) were put into the Eppendorf PCR tubes which were amplified using a machine. The amplification was carried out including thermal cycler predenaturation at 95°C for 1 min followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Gene amplification products were interpreted using agarose gel electrophoresis at 100 V and 250 mA for 35 min. The electrophoresis results were read on an ultraviolet transilluminator machine (Lumina, India), and each band that appeared was compared with a DNA marker ladder.

Results

MCA was used to identify K. pneumoniae from 150 fresh bat fecal samples. Samples were taken from the Tanjung Ringgit bat cave, East Lombok. K. pneumoniae colonies have a characteristic smooth, convex surface, mucoid, and pale pink color, as shown in Figure-1, and microscopic analysis revealed Gram-negative bacteria, as shown in Figure-2. Antibiotic resistance test results using the Kirby–Bauer method were characterized by the presence of an inhibitory zone around the antibiotic disk for bacterial growth. This study showed that 21% (3/14) samples were multidrug resistant to CAZ, AML, STR, and CIP in samples codes B23, A46, and B40. As many as 50% (7/14 K. pneumoniae isolates) were counted as multidrug resistant to AML, STR, and CIP in sample codes A23, B54, B12, B46, B14, B1, and B44. The size of the inhibition zone for CAZ was 17 mm, AML 13 mm, STR 11 mm, and CIP 21 mm. Minimum inhibitory concentration or inhibition zone diameters were to determine resistance patterns and detect the presence of MDR in bacterial isolates using the 2020 clinical and laboratory standard institute (CLSI), as shown in Tables-2 and 3. The results of this study showed that ten isolates of K. pneumoniae were isolated from fresh bat feces in the Tanjung Ringgit cave, East Lombok, which were MDR bacteria. MDR is the occurrence of antibiotic resistance in isolates that are resistant to three types as shown in Figure-3.

The results of this study showed that 60% of MDR K. pneumoniae isolates samples tested by PCR were positive for blaSHV detection. Detection of blaSHV as an ESBL gene indicates that K. pneumoniae isolates isolated from fresh bat feces from the Tanjung Ringgit bat cave in East Lombok can produce ESBL, as shown in Figure-4.

The results showed that 14/150 (9.3%) fresh bat feces samples taken from the Tanjung Ringgit bat cave, East Lombok, were positive for K. pneumoniae. The antibiotic resistance test using the Kirby–Bauer method was characterized by the following presence of an inhibitory zone around the antibiotic disk for bacterial growth. This study showed that 21% (3/14) of samples were MDR to CAZ, AML, STR, and CIP, and 57% (8/14) were resistant to AML, STR, and CIP. As many as 100% of K. pneumoniae isolates were resistant to AML (Table-3).

Discussion

Numerous reports of an increase in K. pneumoniae have shown MDR including carbapenems, ESBL, and fluoroquinolones that resulted longer treatment duration and are difficult to cure [2]. The existence of antibiotic resistance is expected patterns that can guide the selection of appropriate antibiotics [12]. In this study, 10 MDR K. pneumoniae isolates had the same resistance pattern to several antibiotics, one of which was resistant to AML with a result of 100% (10/10). AML is a broad-spectrum penicillin antibiotic that was first discovered; therefore, it is often used by the public both in humans and animals to cure various diseases such as urinary tract infections, bronchitis, pneumonia, and laryngitis [13]. Based on research conducted by Puspitasari et al. [14], as many as 89.4% (107/120 people) had used AML as an antibiotic as treatment in Ampenan District, Mataram City, West Nusa Tenggara Province, Indonesia. The low level of public knowledge regarding the use of antibiotics requires supervision and education.

ESBL produced by bacteria destroys the beta-lactam ring found in penicillin antibiotics. This enzyme can be found innately on the bacterial chromosome or is obtained through plasmid [15]. As many as 90% of cases of disease caused by K. pneumoniae occur in intensive care unit patients due to AML resistance in hospitals in northern India [16]. The rapid development of K. pneumoniae strains worldwide that are resistant to almost all beta-lactam antibiotics, including carbapenems, indicates an organism’s rapid response to certain environmental changes [16].

Table-1: Primers were used.

<table>
<thead>
<tr>
<th>Gene targeted</th>
<th>Primers</th>
<th>Base pair</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHV</td>
<td>F: 5’-GGTTATGCCTATATTGCCTCGCC-3’</td>
<td>867 bp</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td>R: 5’-TTAGGTTGCAGTTGCTC-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SHV=Sulfehdryl-variable
Ten *K. pneumoniae* isolates in this study showed 100% (10/10) resistance to aminoglycoside antibiotics (STR). This resistance may be related to the effectiveness of using STR for the treatment of animals and humans in Indonesia. Kerantzas and Jacobs [17] stated that giving a combination of penicillin and STR antibiotics could be a good treatment, but the use of STR in the treatment of animal diseases needs to be monitored. *K. pneumoniae* that is R to aminoglycoside antibiotics can be caused by changes cellular permeability caused by changes in AcrAb-ToIC and KpnEF efflux pump systems and loss of KpnO porin [16]. This result may indicate different affinities of the apparatus with different aminoglycosides. Direct involvement in permeability aminoglycoside resistance caused by the missing KpnO porin was reported *in vitro* resistant to tobramycin, STR, and spectinomycin [18]. *K. pneumoniae* isolate in this study was also 100% resistant to CIP, which is a fluoroquinolone class of antibiotics(10/10). Since 1998, fluoroquinolones have been classified according to classification by the WHO as critically important in human medicine due to their importance in treating infections caused by *Campylobacter* spp., *Salmonella* spp., and *E. coli*. To prevent further resistance treatment, the use of fluoroquinolones is limited to individual treatment and not to groups.

The use of this antibiotic has been banned on livestock farms even in European countries [19]. As many as 3/10 MDR *K. pneumoniae* isolates were resistant to cephalosporin (CAZ) antibiotics. Cases of resistant to third generation cephalosporin antibiotics such as CAZ can make treatment in humans difficult and take longer. Bacterial sensitivity test to CAZ is also used as a screening test for ESBL bacteria (CLSI 2018) that are resistant to CAZ indicates ESBL-producing bacteria. However, the results of this study showed low resistance. Based on research conducted by Do Tran et al. [20] regarding *K. pneumoniae* isolated from *K. pneumoniae* infection patients at Can Tho General Hospital and Can Tho Central General Hospital, Vietnam, showed that 67.5% (100 samples of *K. pneumoniae* isolates) were resistant to CAZ.

Antibiotic resistance may occur due to inappropriate and excessive use of antibiotics. *Klebsiella* resistant to antibiotics occurs because this bacterium has the ability ESBL enzyme. Resistance genes may originate from bacterial plasmids. The gene responsible for the production of the ESBL enzyme is centered on a plasmid that develops into a point mutation resulting in a change in the configuration of the active part of the original gene and is known as beta-lactamase [21]. The beta-lactamase
enzyme can protect against Gram-negative bacteria against beta-lactamase antibiotics. Target of attack by beta-lactam is the cell wall. This class of antibiotics has a beta-lactam group, similar to the cell walls, which react with enzymes during cell wall formation. The enzyme will no longer function so that the cell wall will not completely form. Cell walls that are not fully formed and bacterial cells without cell walls cause death [22].

Food-producing animals, pets, and wild animals can spread bacteria that are resistant [7, 23, 24]. Resistant bacteria contained in animal waste can migrate through feces around the animal’s living environment, farms, slaughterhouses, including workers who were infected with resistance bacteria with a high potential for spreading these bacteria to the environment or people around them that have been contaminated by bacteria and may have passed through the air during animal transportation [25]. There is ESBL-producing K. pneumoniae isolated from humans, livestock, wild animals, and non-clinical isolates [22, 26].

In research conducted by Pishtiwan and Khadija [9], detection of blaSHV was K. pneumoniae bacteria isolated from patients with thalassemia in Erbil, Iraq with a 35.2%. The blaSHV gene was more frequently detected in K. pneumoniae than S. aureus. This gene is mediated by transposons, plasmids, or chromosomes, which has spread sporadically throughout the world. The prevalence of ESBL-coding genes is high observed in K. pneumoniae and E. coli indicates that MDR is a phenomenon which is commonly found today [9]. The ESBL gene encoding blaSHV is a nosocomial Enterobacteriaceae plasmid gene that has been found in many wild animals in recent [27].

Research conducted by McDougall et al. [2] regarding the novel Klebsiella africana K. pneumoniae strains isolated from Australian fruit bats, blaSHV gene is an ESBL-encoding gene found in 50% of K. pneumoniae isolates (15/30). The presence of the chromosomal SHV beta-lactamase gene causes Klebsiella infection resistant to ampicillin and AML. The blaSHV gene is a plasmid-borne gene that is often found in K. pneumoniae and has spread widely in the Enterobacteriaceae family of bacteria [2].

Research conducted by Nimnoi and Pongsilp [28] in the Upper Gulf of Thailand revealed that 25% of the blaSHV genes were detected in K. pneumoniae bacteria isolated from seawater (12/48). The Klebsiella bacteria that were isolated in this study were resistant to at least one types of antibiotics, including penicillin, CAZ, carbapenems, monobactam aminoglycoside, and phenicol groups. The discovery of Enterobacteriaceae carrying genes encoding beta-lactamases was mediated by plasmids of the ampC, blaSHV, and blaTEM genes. Environment can increase public health risks and can become a public health concern surveillance issues [28].

TEM, SHV, and CTX-M-derived ESBL enzymes were found in Enterobacteriaceae, namely, K. pneumoniae bacteria, which are MDR capable of ESBL production [29]. The TEM and SHV genes are almost always found in E. coli and K. pneumoniae. SHV is very common in K. pneumoniae and constitutes 20% of plasmid-mediated ampicillin resistance and

Table-3: Antibiotics resistant profile of Klebsiella pneumoniae.

<table>
<thead>
<tr>
<th>Code</th>
<th>AML</th>
<th>TS</th>
<th>CIP</th>
<th>STR</th>
<th>CAZ</th>
<th>MDR</th>
<th>blaSHV</th>
</tr>
</thead>
<tbody>
<tr>
<td>B23</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A46</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B40</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A23</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>B54</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B127</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B46</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B143</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B12</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B44</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

AML=Amoxicillin, TS=Trimethoprim-Sulfametoxazole, CIP=Ciprofloxacin, CAZ=Ceftazidime, STR=Streptomycin, MDR=Multidrug resistance, R=Resistant, S=Sensitive, + =Positive, - =Negative

Figure-4: Detection of bla-sulphydryl-variable gene encoding extended-spectrum beta-lactamase on Klebsiella pneumoniae from fresh feces of bats on Tanjung Ringgit Cave, Lombok island, Indonesia.
is integrated in the chromosome [30]. The SHV and TEM have been reported to be detected in wild animals, particularly SHV-12 and TEM-52. The type of sample enzymes encoded by SHV is SHV-102, SHV-1, and SHV-2. TEM-type enzymes TEM-19, TEM-40, TEM-176, and TEM-20 have also been reported sporadically in wild animals [31].

Current public health concern is antibiotic resistance against ESBL producing bacteria [32]. According to the Ambler classification, ESBL is included in Class A with SHV, one of the coding genes. This gene is found most frequently in K. pneumoniae and is responsible for 20% of plasmid-mediated ampicillin resistance. This type of ESBL is commonly found in Europe and the USA, but is currently found throughout the world [33]. ESBL expression ESBL mediates resistant to broad-spectrum CAZs such as ceftriaxone, cefotaxime, and aztreonam [21]. SHV beta-lactamase is able to hydrolyze CAZ, all expanded-spectrum CAZs, and other beta-lactam antibiotics [27]. The presence of an ESBL gene can be detected in isolates of animal origin, although this class of antibiotics has never been used in animals [34]. In this study, the gene encoding ESBL could be detected in 1 isolate of K. pneumoniae that was resistant to three classes of antibiotics (MDR).

The presence of the ESBL-coding gene indicates the spread of resistance genes in humans to the animals bacteria, particularly K. pneumoniae, which is MDR, pose a threat to public health and livestock [35]. The existence of resistance leads to limited treatment options. The previous research on the detection of bacteria that have MDR properties and ESBL resistance genes, although the origin of bacterial resistance genes is unclear wild animals, remains unclear because they are not exposed directly to antibiotics [36].

Therefore, environmental contamination originating from feces, water, and soil containing antibiotic-resistant bacteria can be a potential source of AMR contamination. Bats are wild creature’s animals with the habit of migrating and flying long distances, even between continents, so they have the potential to become a reservoir for MDR bacteria capable of ESBL production. Thus, new routes of MDR transmission and is partially responsible for the global spread of antibiotic resistance [31]. Contamination of foods and water by wildlife, especially bats, is recognized as an important risk factor for the transmission of pathogens or antibiotic resistance to both humans and animals [37]. On the basis of this phenomenon, the role of all parties, as in the One Health concept, is needed to prevent the spread of MDRs from becoming more widespread. One Health approach is an approach that discusses human, animal, and environmental health [38]. Moreover, Human Health is closely related to the health of animals and the environment. The One Health Solution approach is implemented using three principles: communication, coordination, and cooperation between human health, animals, the environment, and other sectors [39]. No one organizations or sectors can solve problems alone, but rather require cooperation and collaborations from all sectors. To prevent the further spread of antibiotic-resistant bacteria, it is hoped that both human and animal health will implement good hygiene as human or animal health and sanitation experts in the surrounding environment will be authorized to carry out treatment measures to better use antibiotics.

Conclusion

K. pneumoniae was found in fresh bat feces taken from the Tanjung Ringgit bat cave, East Java, Indonesia Lombok, as much as 9.3% (14/150). As many as 10/14 samples were positive for K. pneumoniae were MDR with a resistant pattern to AML, CAZ, CIP, and STR. The MDR isolates were followed by ESBL molecular detection of gene using PCR, and it was confirmed that six samples were positive for the blaSHV gene. The detection of K. pneumoniae bacteria encoding ESBL from fresh bat feces indicates the need to monitor the use of antibiotics in humans and animals.

Authors’ Contributions

KNK and YRM: Collected the samples, laboratory works, and drafted the manuscript. OSMS and ALDA: Analysis and interpretation of data. YP: Concept and design of the study. YKKW and WT: Analysis and interpreted the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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