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Molecular detection of extended spectrum β-lactamase producing *Klebsiella pneumoniae* from wild deer

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Abstract. *Riwu KHP, Rantam FA, Effendi MH, Khairullah AR, Widodo A, Kurniawan SC, Ugbo EN. 2022. Molecular detection of extended spectrum β-lactamase producing* Klebsiella pneumoniae *from wild deer. Biodiversitas 23: 4256-4262.* Antimicrobial resistance (AMR) has become a severe threat worldwide and its remote cause remains unclear in most parts due to the global emergence of new resistance mechanisms by microorganisms. This research aimed to identify the presence of extended-spectrum β-lactamase (ESBL) *Klebsiella pneumoniae* from wild deer feces in several places in East Java and East Nusa Tenggara. This study used 129 fresh feces samples collected from wild deer and analyzed for the presence of *K. pneumoniae* using standard microbiological techniques. An antibiotic sensitivity test was performed using Kirby-Bauer Disc Diffusion Method. The isolates were confirmed to the ESBL producer by double disk test and ESBL genes were identified by PCR. Nine samples (6.98%) were *K. pneumoniae* positive out of the 129 wild deer fresh feces. Among the nine *K. pneumoniae* isolates studied 3 (33.33%) were multidrug resistant, East Java (1 isolate) and East Nusa Tenggara (2 isolates). All the multidrug-resistant *K. pneumoniae* isolates were ESBL positive and also harbored *bla*TEM gene. The presence of MDR, and ESBL-producing *K. pneumoniae* isolates was confirmed in wild deer and this explained that wild deer should be considered a source of transmission of MDR and ESBL to public health.

Keywords: *bla*TEM gene, ESBL, *Klebsiella pneumoniae*, MDR, public health, wild deer

INTRODUCTION

Klebsiella pneumoniae is a major cause of sepsis that is predominantly linked to healthcare-associated illnesses. Due to the rise of multidrug-resistant *K. pneumoniae*, new approaches are required to prevent or treat infection. Most human infections are caused by *K. pneumoniae*, which is also one of the most frequent bacteria that induce multidrug resistance (MDR) globally (Santajit and Indrawattana 2016). Septic infections can be caused by *K. pneumoniae*, which can be found in people's digestive, urinary, and respiratory tracts (Bradley and Scoular 2019). It's also present in a variety of places, including soil, water, and plants. *Klebsiella pneumoniae* is prevalent in a variety of domestic and wild mammals, as well as insects, and has been detected in food (Guo et al. 2016).

In animals, *K. pneumoniae* is responsible for pneumonia, epidemic cervicitis and cervicitis in horses, and sepsis in foals. *Klebsiella pneumoniae* is generally associated with bovine pneumonia and mastitis (Saishu et al. 2014), reduced milk yield, poor milk quality, and high mortality in infected cattle (Hertl et al. 2014). Infection can result in significant financial losses and losses to the dairy industry, even on well-managed dairy farms (Paulin et al. 2008). Studies of *K. pneumoniae* in veterinary medicine are still very rare, and the risk of human transmission after contact with animals and ingestion has not been studied at all. Little information has been isolated from non-human sources regarding the effects of *K. pneumoniae* infection on livestock welfare and productivity, epidemiology, resistance profiles, and evolution of resistance.

The rapid spread of antimicrobial resistance microorganisms has increased the isolation level of the *K. pneumoniae* MDR strain in humans. Several strains with different antimicrobial resistance (AMR) gene patterns have been isolated from several European countries (RKI 2013). According to data from the antimicrobial resistance monitoring system ARS, *K. pneumoniae* MDR in the population has increased dramatically in recent years (Remschmidt et al. 2017). The results of clinical trials improved over time (Eckmanns et al. 2014). *K. pneumoniae* is highly resistant to a wide range of antibiotics, including β-lactam antibiotics, fluoroquinolone antibiotics, and aminoglycoside antibiotics (Fair and Tor 2014; Dsouza et al. 2017). Transmission in a bacterium from one host to another through the transmission of a resistance gene present in a

cellular genetic material between bacterial species, including the transmission of clones or horizontal infection of genes. This process is influenced by the use of antibiotics in human and veterinary medicine (Riwu et al. 2020).

Currently, multidrug resistance (MDR) *K. pneumoniae* infections are also a major problem because of their higher morbidity, longer hospital stays, higher mortality rates, and too high medical costs compared to antibiotic-related infections. It has become due to susceptible microorganisms (Correa et al. 2013; Ma and Wang 2013). There have been reports of contamination of food animals or food products with MDR *K. pneumoniae* (Davis and Price 2016; Wu et al. 2016; Projahn et al. 2019). It was also reported that the presence of *K. pneumoniae* in MDR chicken meat in Algeria (Chaalal et al. 2020). Similarly, another high-risk MDR clone has been detected in animals in China (Yang et al. 2019). *Klebsiella pneumoniae* is an opportunistic pathogen that can infect humans and animals and is a common contaminant of retail meat, the increasing prevalence of MDR with virulence poses a potential threat to food safety, and animal and human health (De Koster et al. 2022).

The proximity of livestock or humans has often been suggested as a driver of extended-spectrum β-lactamase (ESBL) produced by *K. pneumoniae*, but in wildlife, to our knowledge, no studies have demonstrated human-to-wild animal transmission or vice versa. To gain a better insight into antibiotic resistance, and the genetic relationship of *K. pneumonia* strains, we performed antibiotic susceptibility testing and phylogenetic analysis of *K. pneumoniae* isolated from wild deer originating from East Nusa Tenggara and East Java, Indonesia for further study of their relationships.

MATERIALS AND METHODS

Sample collection

A total of 129 samples of fresh wild deer feces, consisting of 89 samples of fresh deer feces from the province of East Java and 40 samples from the province of East Nusa Tenggara. All fresh samples were collected and put into buffered peptone water media and each sample was labeled and explained accordingly. In the sample transportation process, all sample specimens were put into a cool box that contains an ice pack, was transported to the laboratory immediately to the Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, Airlangga University Surabaya, and Gastroenteritis Laboratory Tropical Disease Center laboratory, Airlangga University Surabaya, Indonesia for proper analysis.

Isolation and identification of *Klebsiella pneumoniae*

The samples suspension from buffered peptone water medium onto the MacConkey agar and sub-cultured to get the pure bacterial isolates. The isolates were then identified based on morphological characteristics which included colony morphology, cell morphology, and Gram staining test. Colony morphology observations were based on the shape, color, and edges of the bacterial colonies. Observations of bacterial cell morphology include the shape and structure of bacterial cells. Each isolate was further characterized using biochemical methods, the biochemical tests carried out were triple sugar iron agar (TSIA), sulfide indole motility (SIM), Urea, Citrate, MR-VP, and carbohydrate fermentation test (glucose, mannitol, maltose, lactose, and sucrose) (Lestari et al. 2016).

Antibiotic sensitivity test

The Kirby-Bauer agar diffusion method was used in antibiotic susceptibility testing (Ningrum et al. 2016). Thus, the obtained regions were then grouped into sensitive (S), intermediate (I), or resistant (R) groups (Sagita et al. 2015; Kusumaningrum et al. 2016). The antibiotic discs used were aztreonam (30 µg), trimethoprim (5 µg), tetracycline (30 µg), streptomycin (10 µg), ciprofloxacin (5 µg). Purified cultures were prepared in suspension with the equivalent of 0.5 McFarland. The inoculum was then removed with sterile cotton, the sterile cotton was dipped in the suspension and spread evenly over the surface of Mueller Hinton medium (MHA), then allowed to stand for ±5 min. Antibiotic-containing dishes were placed on pure MHA cultures at a distance of 25-30 mm and then incubated at 35°C for 24 h (Masruroh et al. 2016). The diameter of the zone of inhibition was measured in millimeters and was interpreted as sensitive, intermediate, and resistant (CLSI 2017).

Confirmation test of ESBL

ESBL-producing *K. pneumoniae* bacteria isolated from fresh deer feces were confirmed by double disc synergy test method (DDST) on Mueller-Hinton agar according to the recommendations of the Clinical Laboratory Standards Institute (CLSI) (CLSI 2017). A confirmation test with the DDST method was carried out to evaluate the presence of an inhibitory zone of ESBL activity with clavulanic acid using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Merck, Germany) (CLSI 2017). Ceftazidime (CAZ; 30 g), cefotaxime (CTX; 30 g), and amoxycillin-clavulanic (AMC; 30 g) were placed on the media in the parallel form at a distance of 15 mm away from each other. The results of the evaluation after incubation showed synergy between cefotaxime/ceftazidime with the combination of amoxycillin-clavulanic in the form of an increase in the inhibition zone of 5 mm between the diameter of the antibiotic plate indicating positive ESBL bacteria *K. pneumoniae* (CLSI 2017; Savira 2014; Putra et al. 2020).

Detection of *bla*$_{TEM}$ gene from ESBL-producing *Klebsiella pneumoniae*

Klebsiella pneumoniae DNA extraction was done using a QIAamp DNA mini kit 50 (Qiagen, USA) according to the manufacturer's protocol. Briefly, samples were added with 5µL lysozyme (5 mg/mL) enzyme and incubated for 30 min at 56ºC. Next, the extracted DNA was diluted to 100µL with a buffer kit. The DNA solution used for PCR amplification was 1µL. Amplification was performed using a Promega GoTag Master Mix according to the manufacturer's protocol with pure *K. pneumoniae* genomic DNA as a template. PCR amplification was performed using ***bla***$_{TEM}$ (F) and ***bla***$_{TEM}$ (R) (Table 1). The

amplification conditions include an initial denaturation process of 1 cycle at 94°C for 2 min, denaturation at 94°C for 1 min, followed by annealing of 30 cycles at 52°C for 30 sec, an extension at 72°C for 40 sec, and final extension of 1 cycle at 72°C for 5 min. The amplification product was separated on 2% agarose gel and stained with gel-red. The stained gel was then visualized using UV light. Detection of the ***bla*TEM** gene was considered positive when a band at 799 bp was observed on gel (Ali et al. 2016; Hasibuan et al. 2018).

Out of the 129 wild deer fecal samples studied, 9 (6.98%) were positive for *K. pneumoniae* isolates, 4 (4.49%) were from East Java and 5(12.50%) were from East Nusa Tenggara (Table 2; Table 3). Thus, the three (33.33%) isolates were MDR and ESBL-producers (Figure 2), in East Java (1 *K. pneumoniae* isolate) and East Nusa Tenggara (2 *K. pneumoniae* isolates). ESBL-producing *K. pneumoniae* isolates were further analyzed molecularly, and all three isolates harbored ***bla*TEM** genes (Figure 3).

RESULTS AND DISCUSSION

Results

MacConkey agar media was used to identify *K. pneumoniae* from fresh deer feces in 129 samples collected from the two provinces. *Klebsiella pneumoniae* colonies were spherical in shape with a soft, moist surface, and reddish pink (Figure 1).

Discussion

The result of this study revealed the presence of multidrug-resistant *K. pneumoniae* and the prevalence were found to be 33.33%, in East Java (1 *K. pneumoniae* isolate (25.00%)) and East Nusa Tenggara [2 *K. pneumoniae* isolates (40.00%)]. However, all the multidrug-resistant *K. pneumoniae* isolates were ESBL positive and also harbored the ***bla*TEM** gene. This is in line with the report that Gram-negative pathogens have developed resistance by developing enzymes that can destroy antibiotics, by having resistance metabolic pathways, and by altering receptors for an antimicrobial agent (Okonko et al. 2009; Effendi et al. 2021). *Klebsiella pneumoniae* has developed two types of antibiotic resistance mechanisms. One mechanism involves ESBL expression, which develops bacterial resistance to cephalosporins and monobactam. The second mechanism involves the expression of carbapenemase, which helps develop resistance to all available beta-lactams (CDC 2015; Permatasari et al. 2020). Certain ecology aspects such as soil, wastewater, animals, and food products are sources of multidrug resistance organisms, such as *K. pneumoniae* and other bacterial organisms. Source of human infections are caused by close contact with blood, saliva, feces, urine, animal feces, and ESBL carrier organisms can be seen in contaminated animals water or food products (Founou et al. 2016; Rahmahani et al. 2020), also report showing that patients (Leverstein et al. 2011; Overdevest et al. 2011), healthy individuals from the community (Vinue et al. 2009; Geser et al. 2012), meat (Overdevest et al. 2011; EFSA2011), livestock (Dierikx et al. 2012; Hartmann et al. 2012), and companion animals (Dierikx et al. 2012) have been found to be infected with ESBLproducing *Enterobacteriaceae.* Transmission between humans and animals can occur through the food chain (Leverstein et al. 2011; Overdevest et al. 2011), contact with livestock (Dierikx et al. 2012), or the environment (Dierikx et al. 2012).

Figure 1. *Klebsiella pneumoniae* isolates onto Mac Conkey Agar plate

Figure 2. Confirmed ESBL-producing *Klebsiella pneumoniae* by Double Disc Synergy Test

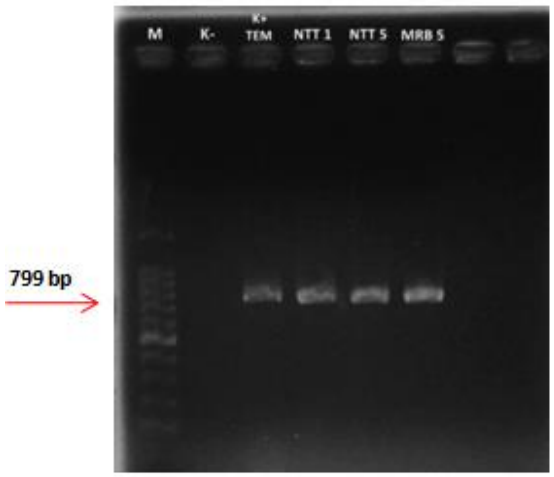

Figure 3. Gel electrophoresis showing the presence of ***bla*TEM** gene in ESBL-producing *Klebsiella pneumoniae* isolates from wild deer feces at 799 bp nucleotide length. Note: M=marker; K-=negative control used *Escherichia coli* ATCC 25922; K+= positive control used *Klebsiella pneumoniae* ATCC 700603; NTT1; NTT5 and MRB5 are ESBL-producing *Klebsiella pneumoniae*

Table 1. The primers used in this study

Gene target	Primer sequence	Amplicon (bp)	Reference
***bla*$_{TEM}$** (F)	CAT TTT CGT GTC GCC CTT ATT TC	799 bp	Dallenne et al. 2010
***bla*$_{TEM}$** (R)	CGT TCA TCC ATA GTT GCC TGA C		

Table 2. Prevalence of extended-spectrum βlactamase (ESBL) producing *Klebsiella pneumoniae* from fresh deer feces

Study province	Sample size	*Klebsiella pneumoniae* (%)	MDR (%)	ESBL (%)	*bla*$_{TEM}$ gene (%)
East Java	89	4(4.49)	1(25.00)	1(25.00)	1(25.00)
East Nusa Tenggara	40	5(12.50)	2(40.00)	2(40.00)	2(40.00)
Total	129	9(6.98)	3(33.33)	3(33.33)	3(33.33)

Note: MDR: Multidrug Resistance, ESBL: extended-spectrum beta-lactamase

Table 3. Antibiotic resistance profiles of MDR and ESBL-producing *Klebsiella pneumoniae* from fresh deer feces

No	Sample code	ATM	W	TE	ST	CIP	MDR	ESBL	*bla*$_{TEM}$
1	MRB 5	R	R	R	R	R	+	+	+
2	NTT 1	R	I	R	R	R	+	+	+
3	NTT 5	R	I	R	R	R	+	+	+

Note: ATM: Aztreonam, W: Trimethoprim, TE: Tetracycline, ST: Streptomycin, CIP: Ciprofloxacin. R: Resistant, I: Intermediate, +: Positive

Human and animal feces are the main sources of ESBL-producing bacteria, where these bacteria get introduced to the environment. People can be affected by these infection-producing bacteria due to recreation on the surface of contaminated fresh water, contaminated use water for drinking, aquatic and marine life, and inhalation of bioaerosols. There has been researched in the past that possible contact with *Salmonella* spp or *Campylobacter* spp in recreational waters is almost the same as the risk of contracting organisms through the consumption of chicken (Wibisono et al. 2020b; Domingues et al. 2012a; Domingues et al. 2012b). There are increasing reports of the contaminated environment with ESBL-producing *Enterobacteriaceae* both in developing and developed countries, in France, a longitudinal survey of various wastewater sources has shown that ESBL-producing *Escherichia coli* was detected in most of the samples, containing *bla*$_{CTX-M}$, at a much higher amount in hospital wastewater than in community wastewater (Brechet et al. 2014; Laupland et al. 2008).

The transfer of pathogens between humans and animals highlights antimicrobial stewardship and symbolically demonstrates the concept of "One Health" (Riwu et al. 2020). Humans, animals, and the environment are interlinked with each other, so the issue of resistance to AMR among them should be focused on equally (Laxminarayan 2013). The source attribution model, which is based on microbes subtype data can help to understand determinants of resistance in humans. The rules behind this methodology are to draw comparisons between genetic profiles of bacteria present in various sources which are found in humans (Barco et al. 2013). They noticed the similarities index among various isolates cannot be based on sequence type, plasmid family, and ESBL gene only, especially when the isolates are not spatially related (de Been et al. 2014). Although sequencing the entire genomes can help to provide an excellent alternative. The question still remains, if it is more appropriate to find the virulence and the content of resistance genes, which undoubtedly provide a genetic backbone. This information can help to track the source of human infection based on bacterial subtypes, distributed in various sources (Barco et al. 2013; Effendi et al. 2018). Sources and pathways involved in the transmission of *Enterobacteriaceae* among humans, and animals have been described in detail, but still, further detail is required. Besides, more details are needed to determine the number of infections caused by specific pathways and sources (Pires et al. 2008; Wibisono et al. 2020a; Effendi et al. 2019).

In this present study, we reported multidrug-resistant *K. pneumoniae* carried by wild deer, suggesting that asymptomatic animal hosts are more likely to be ignored and may become an important reservoir for drug-resistant pathogens; this is in agreement with the observation of Perron et al. (2008) in their study on wild deer. Asia is one of the epicenters of antibiotic resistance, there are a surprising number of drug-resistant strains, including *K. pneumoniae*, and a large number of acquired Gram-negative MDR strains have been found (Jean and Hsueh 2011). AMR can be disseminated rapidly through a variety of possible pathways, including foodborne pathogens, insects, wastewater, pets, food producers or wild animals (Yang et al. 2019; Ansharieta et al. 2021). In particular, more than 25 outbreaks of human infectious disease

outbreaks were reported, 11 associated with animals on farms, and zoos from 1990 to 2000 (Bender and Shulman 2004). Humans can be infected by contacting wild animals such as deer directly or indirectly in some of the interactive activities of the zoo, but this situation is easy to overlook. The role of wildlife at zoos in drug resistance (MDR) epidemiology is of little concern. Several previous studies have shown that bacterial isolates from wild plants and animals living in areas of human activity exhibit stronger drug resistance than wild animals living in remote areas (Allen et al. 2011). As part of human urban life, captive wild animals in the zoo are definitely closer to humans. Therefore, captive animals in zoos can be potential natural reservoirs for AMR and antibiotic-resistant bacteria.

Our results showed that MDR, ESBL, and *bla*$_{TEM}$ gene were confirmed from *K. pneumoniae.* This is similar to the report that some Gram-negative bacteria exhibit MDR and ESBL and the development of resistance is not a new phenomenon and is a worldwide problem (Overdevest et al. 2011; Kristianingtyas et al. 2020; Wang et al. 2020). This study also confirmed the low prevalence of ESBL-producing *K. pneumoniae* from wild deer (33.33%), and this could be a result of low exposure to antibiotics. The observation of all the three isolates of *K. pneumoniae* being MDR, ESBL-producers is in agreement with the study of Wibisono et al. (2020c), which stated that all ESBL-producing isolates were also MDR isolates isolated from poultry animals. The multidrug resistance nature of these isolates can be explained by the fact that ESBLs are mediated by plasmids that carry multi-resistance genes by plasmids, transposons, and integrons, and also they are readily transferred to other bacteria, not of the same species.

In conclusion, this research confirmed the presence of MDR, ESBL, and *bla*$_{TEM}$ genes in *K. pneumoniae* isolated from fecal samples of wild deer at East Java (1 *K. pneumoniae* isolate) and East Nusa Tenggara (2 *K. pneumoniae* isolates) in Indonesia with the total prevalence of 33.33%. Notably, all three *K. pneumonia* isolates harbored the *bla*$_{TEM}$ gene. These results indicate that MDR andESBL-producing *K. pneumoniae* occur in wild animals, therefore this event can cause antimicrobial resistance to other wild animals and human health. This study confirms that there is a close relationship between drug-resistant strains carried by wild animals. Wild animals such as deer may be important reservoirs for clinical isolates of MDR, which pose a potentially serious public health risk. This potential should not be ignored and monitoring of this environment is critical in preventing major threats to public health in the future.

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