BIODIVERSITAS
Volume 24, Number 11, November 2023
Pages: 6092-6099

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d241130

Identification of *bla*SHV and *bla*TEM extended spectrum beta-lactamase genes in *Klebsiella pneumoniae* in the dairy wastewater, East Java Province, Indonesia

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Manuscript received: 13 October 2023. Revision accepted: 20 November 2023.

Abstract. *Dameanti FNAEP, Yanestria SM, Effendi MH, Plumeriastuti H, Tyasningsih W, Ugbo EN, Sutrisno R, Safri MAAS. 2023. Identification of* bla*SHV and* bla*TEM extended spectrum beta-lactamase genes in* Klebsiella pneumoniae *in the dairy wastewater, East Java Province, Indonesia. Biodiversitas 24: 6092-6099. Klebsiella pneumoniae* infection in dairy cows causes various livestock production hazards and threatens public health because it acts as a Multi-Drug Resistance Bacteria (MDRB), especially Extended-Spectrum Beta-Lactamase (ESBL) strains. Livestock wastewater is a repository for commensal isolates including *K. pneumoniae* and potentially can be a reservoir for ESBL spreaders for the environment. The aim of this study was to identify the *bla*SHV and *bla*TEM ESBL genes in *K. pneumoniae* from wastewater of dairy cattle farms in East Java Province. This study used a sample of 342 dairy farm wastewater isolated on 2% BPW media from 6 cities/regencies with the highest population of dairy cows in East Java, Indonesia, namely Pasuruan District, Malang District, Tulungagung District, Blitar District, Batu City and Kediri District. Samples were identified on MCA media, Gram staining, EMBA, and biochemical tests. Confirmation of ESBL phenotypically used the Double Disc Synergy Test (DDST) method and the Polymerase Chain Reaction (PCR) test to detect the ESBL gene. The result showed that bacterial isolation and identification were 14.32% (49/342), confirmed *K. pneumoniae*. DDST confirmation results of ESBL-producing *K. pneumoniae* isolates from the wastewater of dairy farms in East Java obtained were 5.55% (19/342). The sample was continued with the PCR test showing positive for the blaSHV gene 63.1% (12/19) and blaTEM 31.57% (6/19). In conclusion, the ESBL gene *bla*SHV and *bla*TEM identification in *K. pneumoniae* isolates, indicate that wastewater from dairy farms has the potential to be a reservoir of ESBL carriers in the environment and pose a threat to public health in East Java Province, Indonesia.

Keywords: Dairy cattle, ESBL, *K. pneumoniae*, public health, wastewater

INTRODUCTION

The environment highly affects the increase in Antimicrobial Resistance (AMR) as a source of acquisition and transfer of antibiotic-resistance genes between bacteria (Sharma and Gautam 2018; Banu et al. 2021). An increase in AMR emerges due to the improper disposal process of animal and human waste which contaminates the environment (Manaia et al. 2018; Ansharieta et al. 2021). Dairy farming in Indonesia is dominated by the traditional livestock system. Therefore, waste disposal ends up in rivers or the environment around the farms (Dameanti et al. 2023). Bacterial contamination in the environment causes enzymes, mediated by the Extended-Spectrum Beta-Lactamase (ESBL) gene in plasmids, conjugated horizontally to the same or different bacterial species. Hence, they are transferred from humans to animals and vice versa, resulting in an increase in the incidence of ESBLs, especially in the community (Newire et al. 2013; Riwu et al. 2020; Silago et al. 2021). *Klebsiella pneumoniae* carriers of the MDR-resistant gene, especially ESBL, have been widely reported worldwide (Wu et al. 2019; Riwu et al. 2022). There are a lot of evidences showing that the environment is the main source of transmission of ESBL-producing organisms (Banu et al. 2021). The presence of ESBL-producing *K. pneumoniae* in the environment is a particular concern, considering the virulence factor, and is considered a major cause of infectious diseases (Widodo et al. 2020).

The World Health Organization considers Multidrug-Resistant (MDR) *K. pneumoniae* as a major global threat (Arafa et al. 2022). *Klebsiella pneumoniae* is a normal organism that lives in the digestive tract so that it can be isolated from feces and is one of the important pathogenic bacteria from animals and humans (Effendi et al. 2018). In addition to excrement, *K. pneumoniae* has been isolated from various samples, namely milk from milk cans, cow's milk affected by mastitis, fertilizer from dairy cow feces to various environments around the farm, namely on the surface of the water, wastewater, soil, plants and the mucosal surfaces of mammals (Cheng et al. 2018; Gelalcha and Dego 2022). *Klebsiella pneumoniae* is the most common bacterium that causes serious respiratory system infections in both humans and animals. *Klebsiella pneumoniae* has a wide spectrum of virulence factors, including O-lipopolysaccharide, adherence factors, capsular antigens, and siderophores which contribute to various environmental conditions so that they become the cause of various infectious diseases, such as pneumonia, urinary tract infections, bacteremia, wound infections and liver abscesses (Effendi et al. 2018; Wareth and Neubauer 2021).

Extended-Spectrum Beta-Lactamase (ESBL) producing organisms are resistant to beta-lactam antibiotics. Beta-lactams are broad-spectrum antibiotics that are commonly used to treat microbial infections, including *K. pneumoniae* infections (Banu et al. 2021). ESBL has been of particular concern to public health worldwide since 1995 due to variants of the TEM gene (*bla*TEM), SHV gene (*bla*SHV), and CTX-M gene (*bla*CTX-M) (El-Mohandes et al. 2022). It has been described that 100 TEM variants have been identified, since its first detection in *K. pneumoniae* and *K. oxytoca* in the 1970s, while SHV has been described as many as 229 variants (Ogefere et al. 2019; Gelalcha and Dego 2022). Recent studies have shown that ESBL-producing bacteria isolated from humans and livestock harbor identical ESBL genes in plasmids. This study shows that ESBL-producing bacteria or ESBL genes/plasmids from livestock have a deep potential to be transmitted to humans (Schauss et al. 2015). In humans and animals, AMR increases the risk of failure of antibiotic therapy and the genetic features of *Klebsiella* spp. Extended-spectrum beta-lactamase producers are critical in treating pathogenic infections (Lee et al. 2021; Arafa et al. 2022).

This study aims to explain the description of the molecular identification of the *bla*TEM and *bla*SHV genes encoding ESBL in *K. pneumoniae* isolates. Based on the potential transmission of ESBL-producing *K. pneumoniae* from farms to the community, it is essential to explain the description of the identification of the ESBL gene and the potential occurrence of wastewater from dairy farms as a reservoir for ESBL-producing *K. pneumoniae*, especially in East Java as the province with the largest population of dairy cows in Indonesia.

MATERIALS AND METHODS

Ethical approval

This study used wastewater from dairy farms from ditches or trenches around the pens. Ethical approval was not required because this study did not involve the use of live animals.

Study area

This study was conducted from June 2022 to July 2023. The research sample was taken in six cities/regencies with the highest population of dairy cows in East Java Province (Pasuruan District, Malang District, Tulungagung District, Blitar District, Batu City and Kediri District). Samples were processed at the Laboratory of Microbiology and Immunology Veterinary, Faculty of Veterinary Medicine, Universitas Brawijaya, Indonesia, and continued to PCR Molecular Testing which was carried out at the Institute of Tropical Disease Center, Universitas Airlangga.

Sample collection

A 100 mL wastewater sample was isolated from the ditches/cages of 342 dairy farms and stored in a centrifuge tube. The sample is then stored in an icebox during sample transportation and brought to the laboratory for further testing (Yanestria et al. 2019).

Isolation and biochemical identification of *K. pneumoniae*

A 5 mL of wastewater was isolated on the same day with a 2% concentration Buffer Pepton Water (BPW; Oxoid, UK) collection process with a ratio (1:1) and incubated at 37°C for 24 hours (Dameanti et al. 2023). Samples from BPW media were then streaked using a round loop on MacConkey Agar (MCA; Oxoid, UK) media and incubated at 37°C for 24 hours. Colonies suspected of *K. pneumoniae* were purified on Eosin Methylene Blue Agar (EMBA; Oxoid, UK) media and incubated at 37°C for 24 hours (Maulana et al. 2021). The isolates were then proceeded to Gram stain following the procedure of Becerra et al. (2016), and biochemical assays IMViC (Indol motlity (SIM; HiMedia, India), Methyl Red (MR) and Voges Proskauer (VP; HiMedia, India), citrate (HiMedia, India)), Triple sugar iron agar (TSIA; HiMedia, India) and urease (HiMedia, India) (Yanestria et al. 2022).

Phenotypic detection of ESBL by double-disk synergy test (DDST)

Klebsiella pneumoniae isolates then proceeded to the DDST test to phenotypically detect ESBL-producing bacteria using amoxicillin-clavulanate as a beta-lactam inhibitor (Drieux et al. 2008; CLSI 2020). Isolates were isolated on Nutrient Agar medium (Oxoid, UK) and incubated for 24 hours at 37°C. The isolate was then suspended with turbidity equal to 0.5 McFarland. The bacterial suspension was then taken using a sterile cotton swab and planted evenly on the surface of the media. The media was left for 15 minutes and an amoxicillin-clavulanic acid disc (AMC; Oxoid, UK) (30/10 µg) was placed in the middle of the media, followed by a ceftazidime disc (CAZ; Oxoid, UK) and cefotaxime disc (CTX; Oxoid, UK) with a distance of 20 mm from the AMC. Cultures were incubated at 35-37°C for 18-24 hours (Putra et al. 2020). DDST results were observed based on CLSI interpretation (2020).

Table-1. Primers of ESBL genes *bla*TEM and *bla*SHV

Gen	Primary sequence (5'-3')	PCR conditions	Application size (bp/*base repair*)
*bla*TEM	F: 5' ATGAGTATTCAACATTTCCG 3' R: 5' CTGACAGTTACCAATGCTTA 3'	1 cycle of 5 min at 96°C; 35 cycles of 1 min at 96°C; 1 min at 58°C; 1 min at 72°C; 1 cycle of 10 min at 72°C	867
*bla*SHV	F: 5' GGTTATGCGTTATATTCGCC 3' R: 5' TTAGGTTGCCAGTGCTC 3'	1 cycle of 5 min at 96°C; 35 cycles of 1 min at 96°C; 1 min at 60°C; 1 min at 72°C; 1 cycle of 10 min at 72°C	867

Identification of *bla*SHV and *bla*TEM genes using Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was performed to detect the presence of the *bla*SHV and *bla*TEM genes in *K. pneumoniae* isolates, which were confirmed by ESBL through the DDST phenotype test. *K. pneumoniae* isolates were re-cultured on Nutrient Agar (NA; Oxoid, UK) and incubated at 37℃ for 24 hours. A pure culture from NA media was taken using a sterile plastic tube and transferred to a PCR tube filled with 1 mL of sterile distilled water and 5 µL of lysozyme (5 mg/mL). The isolated samples were incubated at 56℃ for 30 minutes. The samples were centrifuged at 1000 rpm for 15 minutes at 4℃ to obtain DNA pellets. The sample DNA pellet was diluted to 100 µL with kit buffer (QIAamp DNA mini kit 50) (Kristianingtyas et al. 2020). The resulting DNA extraction samples were mixed with each DNA primer, Taq DNA polymerase enzyme, deoxyribonucleotide triphosphates and buffers from Thermo Fisher Scientific Inc. (Massachusetts, USA) for the amplification process (Imasari et al. 2018; Kristianingtyas et al. 2020). Details of the primers and amplification process for each gene were presented in Table 1, referring to Ferreira et al. (2011). The *bla*TEM amplification procedure was an initial denaturation of 96℃ for 1 minute, followed by 35 cycles consisting of 96℃ for 1 minute (denaturation), 58℃ for 1 minute (annealing) and 72℃ for 1 minute (extension), and followed by a final extension at 72℃ for 10 minutes. The *bla*SHV amplification procedure was an initial denaturation of 96℃ for five 5 minutes, followed by 35 cycles consisting of 96℃ for one 1 minute (denaturation), 60℃ for 1 minute (annealing), 72℃ for 1 minute (extension) and followed by a final extension at temperature 72℃ for 10 minutes (Imasari et al. 2018). PCR results were visualized by electrophoresis using 2% agarose gel (Invitrogen, USA) (Wibisono et al. 2020). The negative control in this test used *E. coli* ATCC 25922, while the positive control used *Klebsiella pneumoniae* ATCC 700603.

RESULTS AND DISCUSSION

Isolation and identification of *K. pneumoniae*

Isolation and identification results confirmed *K. pneumoniae* of 14.32% (49/342) from 342 total samples of dairy farm wastewater in East Java. These incidents were spread across cities/regencies in East Java, namely Kediri 8.16% (4/49), Blitar 16.33% (8/49), Malang 34.69% (17/49), Batu City 8.16% (4/49), Pasuruan and Tulungagung 16.33% (8/49) as shown in (Table 2). The identification used MCA in Figure 1A EMBA media in Figure 1B and Biochemical test for *K. pneumoniae* were used SIM, TSIA, MR-VP, Citrate and Urease test in Figure 2.

ESBL-producing *K. pneumoniae* confirmation using DDST and PCR test

The results of the incidence of ESBL-producing *K. pneumoniae* using the DDST test, 5.55% (19/342), as in Table 2 spread over the City/District, namely Kediri 0% (0/19), Blitar 26.32% (5/19), Malang 21.05 % (2/19), Batu City 10.53% (2/19), Pasuruan 26.32% (5/19) and Tulungagung 15.79% (3/19). The evaluation results were positive after incubation, which showed synergy between CAZ/CTX and AMC through an increase in the inhibition zone ≥5 mm between the diameters of the antibiotic discs as shown in Figure 3 (CLSI 2020; Wibisono et al. 2021). Confirmation results for the existence of the *bla*SHV and *bla*TEM genes through PCR tests, namely *bla*SHV 63.1% (12/19) and *bla*TEM 31.57% (6/19). These incidents scattered from various cities/regencies, namely Blitar 33.33% (4/12) with 2 positive isolates *bla*SHV and *bla*TEM, 2 isolates of *bla*SHV and 1 isolate of *bla*TEM only; Malang 8.33% (1/12) with 1 isolate positive for *bla*SHV and *bla*TEM; Batu City and Pasuruan both had 25% (3/12) with 1 positive *bla*SHV or *bla*TEM isolate and 2 *bla*TEM isolates; and Tulungagung 8.33% (1/12) with 1 isolate positive for *bla*SHV only. PCR molecular identification showed visualization of the *bla*TEM gene fragment band with a gene length of 867 bp as shown in Figure 4, while the *bla*SHV gene with a gene length of 867 bp is shown in Figure 5.

Discussion

The results of isolation and identification of *K. pneumoniae* were spread over cities/regencies, namely Kediri 8.16% (4/50), Blitar 16.33% (8/55), Malang 34.69% (17/63), Batu City 8.16% (4/59), Pasuruan 16.33% (8/61) and Tulungagung 16.33% (8/54). The identification results of *K. pneumoniae* were based on bacterial characteristics, which showed lactose fermented and mucoid results on MCA media as shown in Figure 1A (Arafa et al. 2022). On EMBA media *K. pneumoniae* showed pink-black colonies, medium-large size with flat edges and mucoid as shown in Figure 1B (El-Mohandes et al. 2022). The results of Gram staining for *K. pneumoniae* were Gram-negative and rod-shaped (Lenchenko et al. 2020). Biochemical test results for *K. pneumoniae* were positive gas, change of media to yellow (acid), and negative of H_2S on TSIA media; nonmotile and negative for indole on SIM and MR tests; while the VP, SCA, and Urease tests showed positive results

as in (Figure 2) (Hansen et al. 2004; Cappuccino and Welsh 2019; Bolla et al. 2021). The highest incidence of *K. pneumoniae* isolation occurred in Malang District, Indonesia. *Klebsiella pneumoniae* is an organism that can be found in aquatic and other environments (Keesler et al. 2020). *Klebsiella pneumoniae* has complex virulence factors, such as O-lipopolysaccharide, adherence factors, and capsular antigens (Arafa et al. 2022). With these virulence factors, *K. pneumoniae* is resistant to various extreme environmental conditions, disinfectants and many classes of antibiotics. Some strains are also capable of producing endotoxins and exotoxins which cause several infectious and deadly diseases (Lenchenko et al. 2020). *Klebsiella pneumoniae* is an opportunistic disease agent in animals and humans from various infectious diseases, including pneumonia, urinary tract infections, skin and intra-abdominal wounds, rhinoscleroma, liver abscess, bacteremia, and atrophy of the nasal mucosa (Newire et al. 2013; Rahamathulla et al. 2016; Gelalcha and Dego 2022). In dairy cattle, *K. pneumoniae* is the most common cause of bovine pneumonia, metritis, and mastitis. In addition, milk can be easily contaminated by farmers and the environment during milking, thus increasing the prevalence of dairy farms in the food chain to the community (Yang et al. 2019). During the last few years, the prevalence of *K. pneumoniae*, especially in livestock (dairy cattle), in the world has not been investigated with certainty (Wareth and Neubauer 2021). However, the results of an isolation study of ESBL-producing *K. pneumoniae* in Indonesia from swab samples of food-producing animals in East Java were found to be 4.61%, while in the feces of residents around dairy farms in Surabaya was 4.2% (Effendi et al. 2018; Imasari et al. 2018).

Extended-spectrum beta-lactamase producing *K. pneumoniae* in the environment has become a concern considering their virulence vector and main causes of infectious diseases (Widodo et al. 2020). The *K. pneumoniae* isolate belongs to the Multidrug Resistance (MDR) bacteria group. *Klebsiella pneumoniae* is resistant to antibiotics by various mechanisms, namely enzymatic degradation or inactivation of antibiotic compounds, changes in membrane permeability, and modifying the target location of antibiotic compounds through mutation of bacterial proteins. *Klebsiella pneumoniae* is mostly reported to have acquired many ESBL enzymes as the main mechanism of defense against antibiotics (Loncaric et al. 2016; Wareth and Neubauer 2021). *Klebsiella pneumoniae* is one of the main "ESKAPE" pathogens that causes public health problems worldwide. ESKAPE pathogenic bacteria, including *Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* sp. (Lohr et al. 2017). The results of research by Surgers et al. (2019), found *K. pneumoniae* isolates with 100% genetic virulence factors, ESBL genes of 93.4%, and resistance to colistin, doxycycline, ciprofloxacin and enrofloxacin. This research also explains that ESBL-producing *K. pneumoniae* can produce biofilms (Surgers et al. 2019).

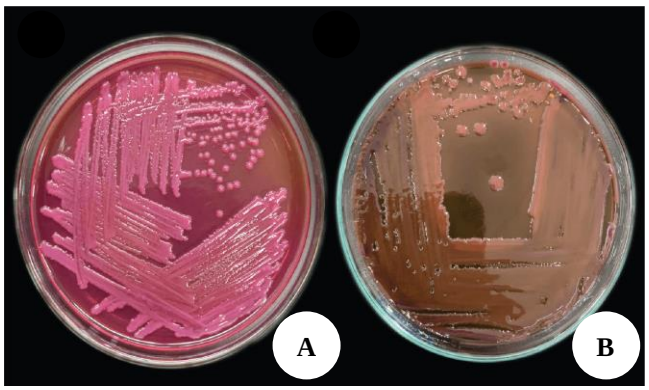

Figure 1. Isolation and identification results of *K. pneumoniae*. A. Suspected *K. pneumoniae* colonies on MCA media; B. On EMBA media

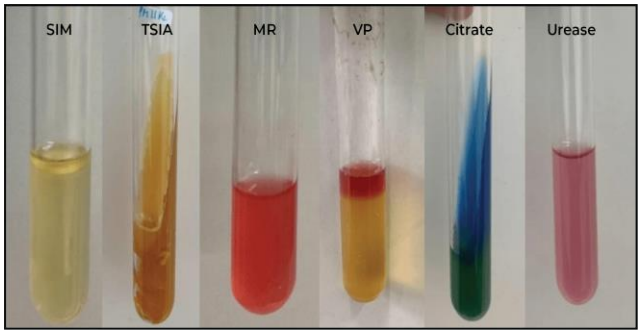

Figure 2. Biochemical test results for *K. pneumoniae* on TSIA, IMViC, and Urease media

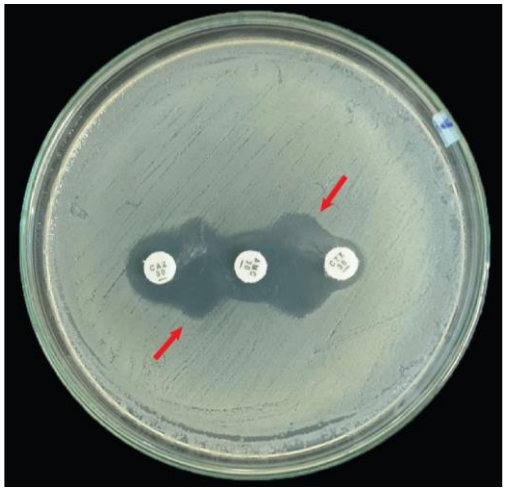

Figure 3. ESBL confirmation results using the DDST method. The results of the evaluation of the increase in the inhibition zone are shown in the red arrows

Table 2. Identification results of ESBL producing *K. pneumoniae* in samples of dairy farm wastewater in East Java

City/District	Isolation and identification		Confirm ESBL with DDST		ESBL confirmation by PCR				
	n	%	n	%	n	SHV	%	TEM	%
Kediri	4	8.16	0	0.00	0	0	0	0	0
Blitar	8	16.33	5	26.32	5	4	33.33	3	50.0
Malang	17	34.69	4	21.05	1	1	8.33	1	16.7
Batu	4	8.16	3	10.53	3	3	25	1	16.7
Pasuruan	8	16.33	4	26.32	3	3	25	1	16.7
Tulungangung	8	16.33	3	15.79	1	1	8.33	0	0
Total	49	100	19	100	13	12	100	6	100

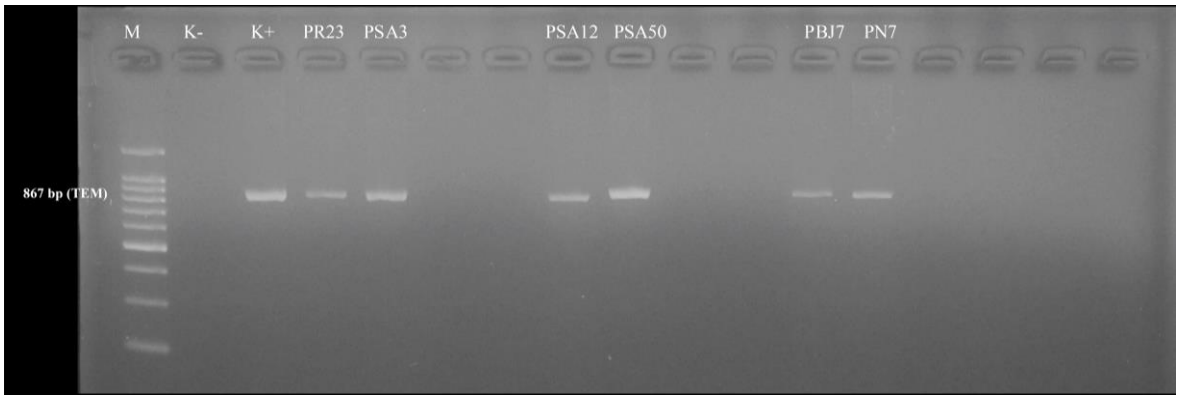

Figure 4. PCR electrophoresis results with visualization of the TEM gene fragment band at a wavelength of 867 bp

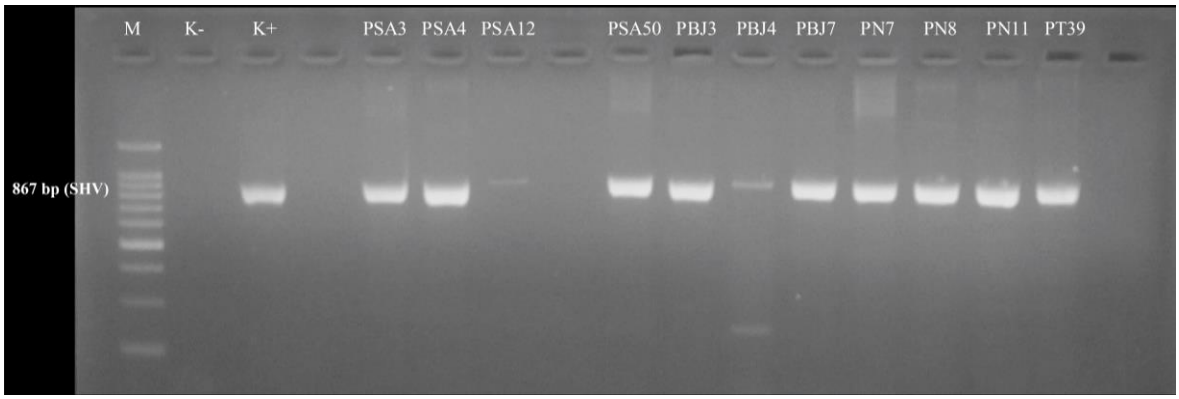

Figure 5. PCR electrophoresis results with visualization of the SHV gene fragment band at a wavelength of 867 bp

Polymerase Chain Reaction test results showed positive for the *bla*SHV gene 63.1% (12/19) and *bla*TEM 31.57% (6/19). These results indicate that the SHV enzyme is more dominant than TEM. Studies suggest that SHV is the most common ESBL type found in a broad range of Enterobacteriaceae (Ghafourian et al. 2015). The *bla*CTX-M, *bla*SHV, and *bla*TEM genes are ESBL genes that are often found in various regions around the world, including human, animal, and environmental isolates. *bla*TEM and *bla*SHV are frequently found in *Escherichia coli* and *K. pneumoniae* (Pehlivanoglu et al. 2016). The study identified that most of the *K. pneumoniae* strains produced *bla*SHV. Meanwhile, *bla*TEM and *bla*CTX-M were identified in *Escherichia coli*, *Enterobacter* spp., and *Klebsiella oxytoca* isolates (Doi et al. 2017; Soekoyo et al. 2020). The majority of ESBL enzymes are derived from the TEM type of the *bla*TEM gene. The *bla*TEM gene is a gene that causes antibiotic resistance and is most frequently detected in clinical populations of Gram-negative microorganisms, including *K. pneumoniae* (Effendi et al. 2018). The results of this study were supported by research from Yuwono (2013), where the *bla*SHV gene was found in 62.50% of *Klebsiella pneumoniae* isolates compared to 21.87% in *Escherichia coli* in RSUP Dr. Mohamad Husein, Palembang. These results are very concerning and demonstrate the risk of transmission of resistant genes to One-Health stakeholders,

including animals, the environment, and communities around the farm. Although the SHV enzyme did not experience a significant spread compared to the CTX-M and TEM variants, in recent years SHV has been found, especially in *K. pneumoniae* isolates with a rising allele variability and in different environmental niches (Liakopoulos et al. 2016). SHV ESBL enzymes are very common in *K. pneumoniae* and account for 20% of the plasmid-mediated ampicillin resistance in this species (Yuwono 2013). The majority of the SHV variants in this reservoir are ESBL types (*bla*SHV-2, *bla*SHV-2a, *bla*SHV-5 and *bla*SHV-12) because they belong to the conjugative plasmid family (IncA/C, IncF, IncHI2) and allow gene transfer/disperse SHV to other nosocomial pathogens that occur in water, namely *S. maltophilia, A. caviae, A. baumannii* and *P. aeruginosa* (Liakopoulos et al. 2016). The first report of SHV-mediated resistance to third-generation cephalosporin antibiotics occurred in 1983 from a plasmid-encoded *Klebsiella* sp. isolate. Furthermore, in a few years, other ESBL variants were identified as plasmid-encoded pBP60, pZMP1, and pUD18 in *K. pneumoniae* from *bla*SHV-2a to *bla*SHV-5 resulting from mutations capable of hydrolyzing broad-spectrum cephalosporins and monobactams (Kliebe et al. 1985). To date, there are more than 246 beta-lactamase TEM-type enzyme derivatives, 229 SHV beta-lactamase derivatives, and 252 variants of the CTX-M enzyme (Gelalcha and Dego 2022).

Most bacterial strains possess the SHV and TEM enzymes contained within the plasmid and are easily transferable to other Gram-negative bacterial species (Ghafourian et al. 2015; Wilopo et al. 2015; Doi et al. 2017). Positive results for the SHV enzyme lead to the potential for horizontal gene transfer between Enterobacteriaceae as a serious public health problem. Contamination of ESBL-producing *K. pneumoniae* allows the ESBL-coding gene on the plasmid and chromosome to be transferred to other bacteria, causing a higher prevalence of ESBL-producing bacteria epidemiologically (Cantón et al. 2012; Biutifasari 2018). It is important to emphasize in this study that the environment and dairy cows play an important role as reservoirs of resistant bacteria and resistance genes. Based on previous studies in Germany, *K. pneumoniae*, which was resistant to gentamicin and trimethoprim was isolated repeatedly from wastewater (Wareth and Neubauer 2021). Previous studies explained that farmers are more likely to be infected with ESBL-producing bacteria than people who do not have direct contact with dairy cows. This incident provides an overview of the spread of ESBL from dairy farms to the community (Price et al. 2007). Therefore, opportunistic pathogens and clinically relevant antibiotic resistance genes in wastewater pose a risk of dispersal to the aquatic environment, thereby spreading to animal populations and to humans. There is a high possibility of transmitting ESBL genes from livestock to humans, so the government needs to develop appropriate interventions and strict policies on antimicrobial use and dairy cattle management to reach a healthier community (Wee et al. 2020).

In conclusion, the identification results of the *bla*SHV 63.1% (12/19) and *bla*TEM 31.57% (6/19) ESBL genes in *K. pneumoniae* isolates show that wastewater from dairy farms has the potential to be a reservoir of ESBL carriers in the environment and pose a threat to public health in Java Province. East, Indonesia. These results can help veterinary authorities overcome antibiotic resistance and environmental pollution caused by wastewater from dairy farms in Indonesia. The government needs to create a new policy regarding wastewater treatment from dairy farms and pollution standards for wastewater from dairy farms that can be released into the environment. This policy was created to create better public health and welfare. It is hoped that these results can create public awareness of the use of antibiotics and wastewater treatment from dairy farms in Indonesia.

ACKNOWLEDGEMENTS

The authors would like to thank the *Penelitian Pascasarjana - Penelitian Disertasi Doktor* funding from *PENELITIAN DRTPM KEMENDIKBUDRISTEK TAHUN* 2023, with grant number from Universitas Airlangga: 741/UN3.LPPM/PT.01.03/2023, the management of KUD Medowo (Kediri District), KUD Jabung, KUD Princi and KUD Poncokusumo (Malang District), KUD Semen (Blitar District), KUD Batu and KUD Junrejo (Batu City), KUD KPSP Setia Kawan (Pasuruan District), KUD Sendang (Tulungangung District) and Laboratory of Veterinary Microbiology and Immunology, Faculty of Veterinary Medicine, Universitas Brawijaya for providing the facilities for the study. Acknowledgments are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

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