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Prevalence of multidrug resistance and extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* from dairy cattle farm wastewater in East Java Province, Indonesia

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Abstract

Background and Aim: Antibiotic resistance in *Klebsiella pneumoniae*, especially extended-spectrum beta-lactamase (ESBL) producers, has become a global public health problem. This study aimed to estimate the prevalence of multidrug resistance (MDR) and ESBL-producing *K. pneumoniae* in wastewater from dairy farms.

Materials and Methods: This study was conducted on dairy farms in East Java Province from June to October 2022. In total, 342 dairy farm wastewater samples were isolated on buffered peptone water media from six cities/regencies with the highest dairy cattle population in East Java. Samples were identified using MacConkey agar media, Gram-staining,eosin-methylene blue agar, and biochemical tests. In total, 14.32% (49/342) samples contained *K. pneumoniae*. Positive isolates were tested for antibiotic sensitivity. *Klebsiella pneumoniae* resistant to beta-lactam was confirmed using the double-disk synergy test to confirm the presence of ESBL-producing bacteria.

Results: The percentage of antibiotic resistance in *K. pneumoniae* was 98% resistance to ampicillin, 67.3% to cefotaxime, 46.9% to tetracycline, 49% to ciprofloxacin, 98% to streptomycin, 14.3% to sulfamethoxazole–trimethoprim, and 83.7% to chloramphenicol. The prevalence of MDR in *K. pneumoniae* was 12.57% (43/342), with the highest prevalence in the five classes of antibiotics at 41.86% (18/43), and the prevalence of ESBL-producing *K. pneumoniae* was 5.55% (19/342), with the highest prevalence in the districts of Blitar and Pasuruan at 26.31% (5/19).

Conclusion: Although the prevalence of ESBL-producing *K. pneumoniae* in wastewater samples from dairy farms was low, caution is recommended because they can be a reservoir for ESBL.

Keywords: animal health, dairy cattle, extended-spectrum beta-lactamase, human health, *Klebsiella pneumoniae*, wastewater.

Introduction

Klebsiella pneumoniae contains a wide variety of virulence factors that cause infectious diseases, such as pneumonia, urinary tract infection, bacteremia, wound infection, and liver abscess [1]. *Klebsiella pneumoniae* infection in livestock causes hazards to livestock production and threatens public health because *K. pneumoniae* is a multidrug-resistant bacteria (MDRB) [2]. The prevalence of *K. pneumoniae* with extended-spectrum beta-lactamase (ESBL), which mediates resistance to third-generation cephalosporins, is increasing worldwide [3].

Poor hygiene and health of dairy cattle cause infectious diseases due to pathogenic bacteria [4, 5]. *Klebsiella*

pneumoniae is one of the most serious threats to public health because it can be transmitted through animals (zoonoses). In dairy cattle, *K. pneumoniae* often causes mastitis [6]. According to Kleinhenz *et al*. [7], the prevalence of mastitis due to *K. pneumoniae* was 14%. Antibiotics are the mainstay of treatment for mastitis caused by *K. pneumoniae* because it shows the prevalence of severe and long-lasting intramammary infection and decreased milk production. Continuous and ineffective use of antibiotics can increase the risk of antimicrobial resistance (AMR) [8]. Bacterial resistance that occurs continuously causes MDRB; therefore, infections from these bacteria are difficult to treat [9].

Klebsiella pneumoniae carrying MDR genes, especially ESBL, has been widely reported worldwide [10, 11]. Extended-spectrum beta-lactamase is an important health problem because its prevalence tends to increase, resulting in high morbidity and mortality [12]. Extended-spectrum beta-lactamase inactivates beta-lactam class antibiotics, such as penicillin; first-, second-, and third-generation cephalosporins; carbapenems; and monobactams, by opening the β-lactam ring [5]. More than 200 types of ESBL have been identified, and there is an increasing prevalence of ESBL, especially TEM, SHV, and cefotaxime (CTX)-M, among *Enterobacteriaceae* in Europe and Asia [13].

Studies have shown that the prevalence of ESBL-producing *Escherichia coli* in wastewater samples from dairy farms in East Java was 22.80% [14]. In addition to *E. coli*, *K. pneumoniae* is commonly found on the surface of water, wastewater, soil, plants, and surfaces of mammalian mucosa [2]. The increase in the prevalence of ESBL is due to the traditional dairy farming system, which enables contamination of the environment through soil and liquid waste [15]. Infiltration of *K. pneumoniae* into the groundwater then spreads to the community through irrigation systems, soil runoff, rainwater, rivers, and water sources [15, 16].

This study aimed to estimate the prevalence of MDR and ESBL-producing *Klebsiella* in wastewater from dairy farms in East Java Province and evaluate the hazard to public health.

Materials and Methods

Ethical approval

Ethical approval was not required because live animals were not used in this study. This study used dairy farm wastewater from dairy cattle from ditches or ditches around the pens.

Study period and location

The research was conducted from June to October 2022. This study used samples of dairy farm wastewater from cities or regencies with the largest population of dairy cattle in East Java Province, Indonesia (Pasuruan Regency, Malang Regency, Tulungagung Regency, Blitar Regency, Batu City, and Kediri Regency). Sampling was conducted after permission from the relevant agency or institution.

Isolation and identification of *K. pneumoniae*

A sample of 100 mL wastewater was isolated using a centrifuge tube from ditches or ditches around the pens. Next, 5 mL wastewater was mixed with 5 mL buffered peptone water (BPW) media at a concentration of 2% (1:1) (Oxoid, UK) and incubated at 37°C for 24 h. Isolates obtained on BPW media were streaked on MacConkey agar (MCA) media (Oxoid) [17]. Pink and mucoid colonies suspected of being *K. pneumoniae* were subcultured on Eosin Methylene Blue Agar (EMBA) (Oxoid) and incubated at 37°C for 24 h [18, 19]. Colonies on EMBA media were subjected to Gram-staining and IMViC tests. Gram-staining involved staining with crystal violet and rinsing with running water, followed by incubation with iodine, acetone alcohol, and safranin. The results of Gram-staining preparations were observed under a microscope at 1000× magnification [20]. Biochemical assays were performed using IMViC (indole motility [SIM; HiMedia, India], methyl red [MR], Voges-Proskauer [VP; HiMedia], citrate [HiMedia], triple sugar iron agar [TSIA; HiMedia], and urease [HiMedia]) [21].

Antibiotic susceptibility test

Antibiotic sensitivity was tested using the Kirby–Bauer disk diffusion method on confirmed *K. pneumoniae* isolates [3]. The antibiotics used were based on journal references and those used in dairy farms in Indonesia. The class of antibiotics used was penicillin/ampicillin, CTX, tetracycline, ciprofloxacin, streptomycin, sulfamethoxazole-trimethoprim, and chloramphenicol (all from Oxoid) [22]. Bacterial isolates were prepared in a suspension with turbidity equivalent to 0.5 McFarland (1.5×10^8 colony-forming unit/mL). Cultures were collected using a sterile cotton swab and spread on the surface of Mueller-Hinton Agar (MHA) media (Oxoid). After approximately 15 min, antibiotic disks were placed on MHA at 25–30 mm and incubated at 35°C for 24 h (Figure-1) [23]. Interpretation of results was recorded

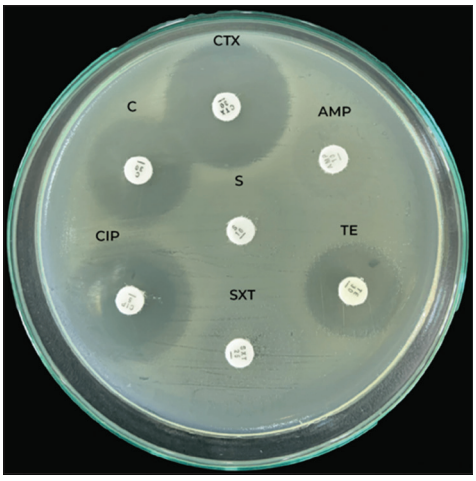

Figure-1: Confirmatory test of antibiotic sensitivity by disk diffusion with the Kirby–Bauer method on Mueller–Hinton agar media.

Table-1: The standard interpretation of antibiotic sensitivity [24].

Antibiotics	Disk content (μg)	Inhibition zone diameter		
		S	I	R
Ampicillin	10	≥17	14–16	≤13
Cefotaxime	30	≥26	23–25	≤22
Tetracycline	30	≥15	12–14	≤11
Ciprofloxacin	5	≥31	21–30	≤20
Streptomycin	10	≥15	12–14	≤11
Trimethoprim-sulfamethoxazole	1.15/23.75	≥16	11–15	≤10
Chloramphenicol	30	≥18	13–17	≤12

according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Table-1) [24]. *Klebsiella pneumoniae* was categorized as MDR when resistant to ≥3 groups of antibiotics [21].

Confirmation of ESBL using the double-disk synergy test (DDST)

Klebsiella pneumoniae isolates positive for MDR were evaluated with the DDST, which is used to phenotypically detect ESBL-producing *Enterobacteriaceae* [25]. This method was performed using the Kirby–Bauer disk diffusion method on MHA media according to recommendations from CLSI [26, 27]. The isolates were inoculated into brain–heart infusion broth (BHIB) (GranuCult, Germany) and incubated for 24 h at 37°C. Isolates in BHIB media were suspended at 0.5 McFarland turbidity. The bacterial suspension was plated evenly on the surface of the media using a sterile cotton swab. After 15 min, an amoxicillin-clavulanic acid disk (Oxoid) (30/10 μg) was placed in the middle of the media, followed by ceftazidime (Oxoid) and CTX (CTX; Oxoid) disks, 20 mm from the amoxicillin-clavulanic acid disk. Cultures were incubated at 35°C–37°C for 18–24 h [28].

Results

Isolation and identification of *K. pneumoniae*

Isolation and identification results confirmed *K. pneumoniae* in 14.32% (49/342) of dairy farm wastewater samples from East Java, including 8.16% (4/50) from Kediri district, 16.33% (8/55) from Blitar district, 34.69% (17/63) from Malang district, 8.16% (4/59) from Batu City, 16.33% (8/61) from Pasuruan district, and 16.33% (8/54) from Tulungagung district (Table-2). On MCA media, *K. pneumoniae* isolates appeared as large mucoid, pink-to-red colonies (Figure-2a). *Klebsiella pneumoniae* ferments lactose and reduces acid to produce pink colonies on MCA media. *Klebsiella pneumoniae* forms mucoid colonies on the media and has a large polysaccharide capsule [18]. Gram-stain results showed that the isolate was Gram-negative (Figure-2c) [29]. Pure colonies suspected of being *K. pneumoniae* on MCA media were plated on EMBA media. *Klebsiella pneumoniae* on EMBA media shows pink-black, mucoid colonies (Figure-2b) [30] with biochemical tests to confirm *K. pneumoniae* which was performed according to the literature as follows: Positive for gas formation, change of media color to yellow (acidic), and negative for H_2S in TSIA media; non-motile and negative for indole in the SIM test; negative on the MR test; and positive (red ring) for VP test, positive (blue) for Citrate test and positive for urease tests [31–33]. The results of the biochemical tests for confirming *K. pneumoniae* are shown in Figure-3.

Table-2: Identification of *K. pneumoniae.*

City/district	Identification of *K. pneumoniae.*	
	n	%
Kediri	4	8.16
Blitar	8	16.33
Malang	17	34.69
Batu	4	8.16
Pasuruan	8	16.33
Tulungagung	8	16.33
Total	49	100

K. pneumonia=Klebsiella pneumoniae

Antibiotic susceptibility test

The prevalence of *K. pneumoniae* resistant to antibiotics was as follows: Ampicillin 98%, CTX 67.3%, tetracycline 46.9%, ciprofloxacin 49%, streptomycin 98%, sulfamethoxazole-trimethoprim 14.3%, and chloramphenicol 83.7%. Detailed AMR results are shown in Table-3 and Figure-4. Bacterial isolates showing resistance to ≥3 groups of antibiotics were categorized as MDRB [21]. The prevalence of *K. pneumoniae* MDR was 12.57% (43/342) (Table-4). Multidrug resistance prevalence was divided into three groups of antibiotics at 16.28% (7/43), four classes of antibiotics at 9.3% (4/43), five classes of antibiotics at 41.86% (18/43), six classes of antibiotics at 27.91% (12/43), and seven classes of antibiotics at 4.65% (2/43).

Confirmation of ESBL using DDST

Double-disk synergy test was used to detect ESBL production by bacteria using amoxicillin-clavulanate as a beta-lactam inhibitor [24]. Positive results were confirmed by an increase in the peripheral zone of cefotaxime ≥5 mm due to clavulanate on the amoxicillin-clavulanate disk diffused with agar and inhibits bacterial beta-lactamases around the ceftazidime disk (Figure-5) [25]. The increase in the zone is caused by clavulanate on the antibiotic disk deactivating ESBL produced by bacteria [34]. The prevalence of

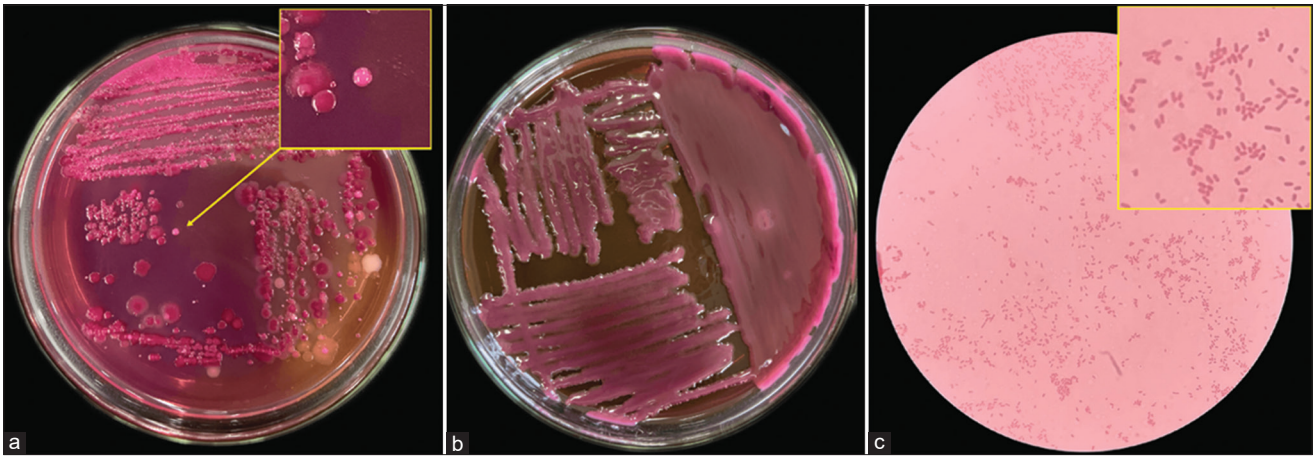

Figure-2: Isolation and identification of *Klebsiella pneumoniae* on MacConkey agar media (a) Eosin Methylene Blue Agar (b) and Gram-staining with 1000× magnification (c). Note: Yellow box as colonies and bacteria suspected to be *K. pneumoniae*.

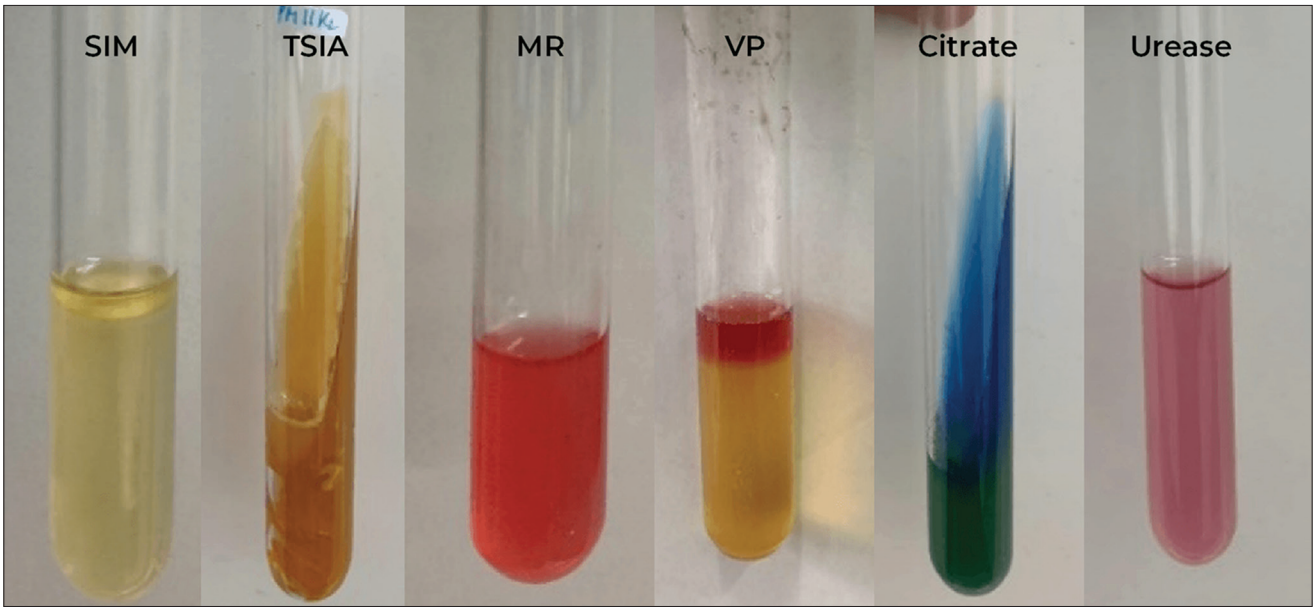

Figure-3: Identification results of *Klebsiella pneumoniae* in biochemical tests. Note: Media SIM, triple sugar iron agar, methyl red, Voges-Proskauer, Citrate, and Urease. Sequentially from left to right.

ESBL-producing *K. pneumoniae* using the DDST test was 5.55% (19/342) (Table-5). These events were spread across cities/regencies, including 0% (0/19) in Kediri, 26.32% (5/19) in Blitar, 21.05% (2/19) in Malang, 10.53% (2/19) in Batu City, 26.32% (5/19) in Pasuruan, and 15.79% (3/19) in Tulungagung.

Discussion

The results showed that the prevalence of *K. pneumoniae* carrying wastewater on dairy cattle farms in East Java was 14.32% (49/342), with the highest prevalence in Malang Regency at 34.69% (17/63). *Klebsiella pneumoniae* is a Gram-negative, non-motile bacterium belonging to the *Enterobacteriaceae* family. *Klebsiella pneumoniae* lives in the digestive tract and can be isolated from feces [1]. In addition, *K. pneumoniae* has been isolated from various samples, such as milk from milk cans, milk from cows with mastitis, rectal swabs of dairy cattle, and manure from dairy cattle. *Klebsiella pneumoniae* is the most common coliform bacteria that cause mastitis in dairy cattle [35]. In addition, *K. pneumoniae* causes metritis, laminitis, and respiratory and digestive diseases in dairy cattle. *Klebsiella pneumoniae* infection is generally treated with antibiotics [36]. In humans, ESBL-producing strains of *K. pneumoniae* are closely associated with urinary tract infection and bacteremia [35]. *Klebsiella pneumoniae* is an opportunistic pathogen because it causes pneumonia, wound infections, urinary tract infection, intra-abdominal and liver abscesses, atrophy of the nasal mucosa, rhinoscleroma, etc. [37]. Although the prevalence of *K. pneumoniae* in wastewater from dairy farms in East Java was only 14.32%, this result should be approached with caution, considering that *K. pneumoniae* causes various diseases in dairy cattle and humans.

All *K. pneumoniae* strains showed significant resistance to ampicillin (98%), streptomycin

Table-3: The results of the prevalence of AMR *Klebsiella pneumoniae*.

AMR data	Kediri		Malang		Blitar		Batu		Pasuruan		Tulungagung		Sub total	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
CTX														
R	1	25	6	35.3	8	100	3	75	7	87.5	8	100	33	67.3
I	3	75	10	58.8	0	0	1	25	1	12.5	0	0	15	30.6
S	0	0	1	5.9	0	0	0	0	0	0	0	0	1	2.0
C														
R	2	50	14	82.4	8	100	4	100	6	75	7	87.5	41	83.7
I	2	50	3	17.6	0	0	0	0	2	25	1	12.5	8	16.3
S	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AMP														
R	3	75	7	100	8	100	4	100	8	100	8	100	48	98.0
I	1	25	0	0	0	0	0	0	0	0	0	0	1	2.0
S	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TE														
R	1	25	5	29.4	3	37.5	3	75	6	75	5	62.5	23	46.9
I	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S	3	75	2	70.6	5	62.5	1	25.0	2	25	3	37.5	26	53.1
S														
R	4	100	16	94.1	8	100	4	100	8	100	8	100	48	98.0
I	0	0	0	0	0	0	0	0	0	0.0	0	0	0	0
S	0	0	1	5.9	0	0	0	0	0	0.0	0	0	1	2.0
SXT														
R	0	0	3	17.6	1	12.5	2	50	0	0.0	1	12.5	7	14.3
I	0	0	0	0	2	25.0	0	0.0	1	12.5	2	25.0	5	10.2
S	4	100	14	82.4	5	62.5	2	50	7	87.5	5	62.5	37	75.5
CIP														
R	0	0	6	35.3	7	87.5	1	25	5	62.5	5	62.5	24	49.0
I	3	75	10	58.8	1	12.5	3	75	3	37.5	3	37.5	23	46.9
S	1	25	1	5.9	0	0	0	0	0	0	0	0	2	4.1

AMR=Antimicrobial resistance, CTX=Cefotaxime, C=Chloramphenicol, AMP=Ampicillin, TE=Tetracycline, S=Streptomycin, SXT=Sulfamethoxazole-trimethoprim, CIP=Ciprofloxacin

Table-4: The results of the prevalence of MDR *Klebsiella pneumoniae.*

Antimicrobial agent	Kediri		Malang		Blitar		Batu city		Pasuruan		Tulungagung		Sub total	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
3	1	14.3	6	85.7	0	0.0	0	0.0	0	0.0	0	0.0	7	16.28
4	0	0.0	2	50.0	0	0.0	0	0.0	0	0.0	2	50.0	4	9.30
5	1	5.6	2	11.1	5	27.8	3	16.7	4	22.2	3	16.7	18	41.86
6	0	0.0	3	25.0	3	25.0	1	8.3	3	25.0	2	16.7	12	27.91
7	0	0.0	1	50.0	0	0.0	0	0.0	0	0.0	1	50.0	2	4.65
Total													43	100

MDR=Multidrug resistance

(98%), chloramphenicol (83.7%), and CTX (67.3%). Consistently, Newire *et al.* [3]. reported that *K. pneumoniae* showed significant resistance to ampicillin and CTX. Beta-lactam antibiotics such as ampicillin (penicillin) and CTX are most often used for humans and cattle in dairy farming [35]. Bacterial isolates resistant to ampicillin and CTX were reported to carry the ESBL gene [35, 38]. A combination of penicillin and streptomycin, chloramphenicol, and a sulfonamide antibiotic is often used to treat infectious diseases in dairy cattle [39, 40]. The high prevalence of resistance in *K. pneumoniae* is caused by the use and misuse of antibiotics; therefore, the bacteria are MDR or resistant to many antibiotics [41]. The misuse of antibiotics leads to selective pressure in bacteria toward evolution as self-defense through genetic mutation, exchange of genetic material, and proliferation [21]. The prevalence of *K. pneumoniae* MDR was 12.57% (43/342), with the highest prevalence found among the five classes of antibiotics at 41.86% (18/43). Multidrug resistance affects the selection of antibiotics for the treatment of bacterial infections. Multidrug resistance causes thousands of deaths annually worldwide [42]. Efforts to reduce MDR include increasing livestock biosecurity and building surveillance programs for feed and livestock. Studies on antibiotic resistance patterns can guide the selection of antibiotics for treating dairy cattle in East Java [43].

Data on ESBL-producing *K. pneumoniae* in wastewater from dairy farms in Indonesia are lacking. The prevalence of ESBL-producing *K. pneumoniae* in wastewater samples from dairy farms in East Java was 5.55% (19/342). This prevalence was considered low compared with studies on ESBL-producing

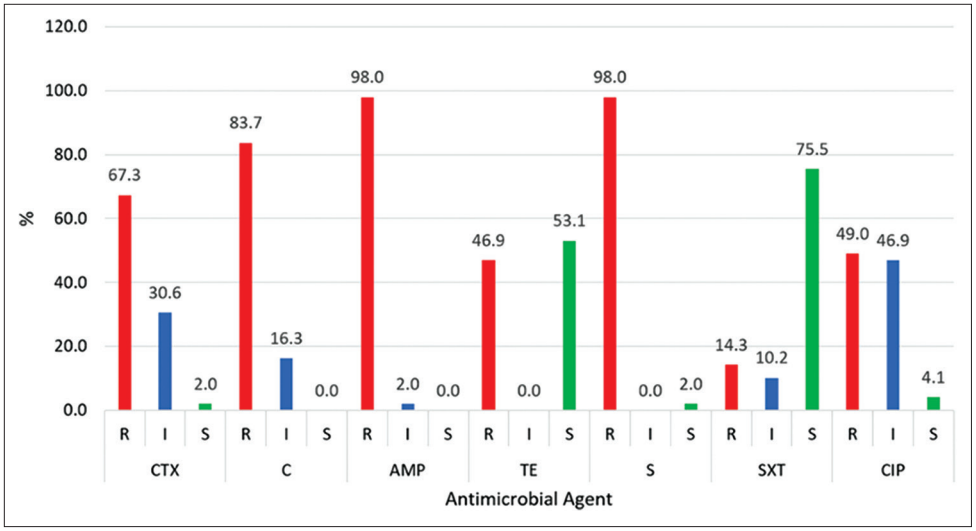

Figure-4: Results of the prevalence of *Klebsiella pneumoniae* AMR against seven classes of antibiotics. CTX=Cefotaxime, C=Chloramphenicol, AMP=Ampicillin, TE=Tetracycline, S=Streptomycin, SXT=Sulfamethoxazole-trimethoprim, and CIP=Ciprofloxacin, R=Resistant, I=Intermediate, S=Sensitive.

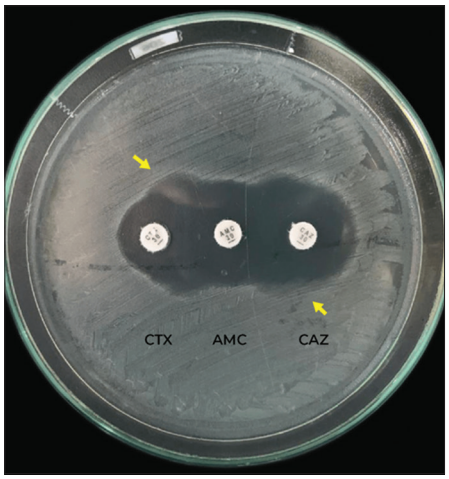

Figure-5: Positive results of extended-spectrum beta-lactamase-producing bacteria on the double-disk synergy test. Note: Yellow Arrows: Peripheral zone increase, CTX=Cefotaxime, AMC=Amoxicillin-clavulanate, CAZ=Ceftazidime.

Table-5: Confirmation results of ESBL-producing *Klebsiella pneumoniae* with DDST test.

City/district	ESBL-producing with DDST test	
	n	%
Kediri	0	0.00
Blitar	5	26.32
Malang	4	21.05
Batu city	3	10.53
Pasuruan	4	26.32
Tulungagung	3	15.79
Total	19	100

ESBL=Extended-spectrum beta-lactamase, DDST=Double-disk synergy test

K. pneumoniae with other isolates in Indonesia and worldwide. The prevalence of ESBL-producing *K. pneumoniae* in Indonesia was ~33.3% [11]. The prevalence of ESBL-producing *K. pneumoniae* was reported to be higher in patients with pneumonia at Sanglah General Hospital, Denpasar (69.2%) and Arifin Achmad Hospital, Pekanbaru (62.5%) in Indonesia [44, 45]. In contrast to studies in humans, the prevalence of ESBL-producing *K. pneumoniae* in swab samples of food-producing animals in East Java was 4.61% [1]. Imasari *et al*. [17] reported an prevalence of ESBL-producing *K. pneumoniae* of 4.2% in the feces of residents around dairy farms in Surabaya. *Escherichia coli* and *K. pneumoniae* are the most common ESBL-producing pathogens. According to a global epidemiological study by Dhillon and Clark [38], the prevalence of ESBL-producing bacteria varies widely worldwide; however, *K. pneumoniae* was the highest ESBL producer in Latin America (44%), the Pacific Rim (22.4%), Europe (13.3%), and North America (7.5%) compared with other bacteria. In Asia, the prevalence of ESBL-producing *K. pneumoniae* isolates continues to increase to >30% [46]. Other studies have reported a 22.80% prevalence of ESBL-producing *E. coli* in wastewater samples from dairy farms in East Java [14].

The increasing prevalence of ESBL-producing bacteria causes ESBL to mutate continuously, leading to the development of new enzymes. Genes or types from TEM, SHV, and CTX-M groups are the most frequently encountered ESBLs [46]. There are >246 TEM beta-lactamase type derivatives, >229 SHV β-lactamase type derivatives, and 252 variants of CTX-M enzyme [35]. Recently, CTX-M variants have increased, particularly in *K. pneumoniae* isolates from Spain, UK, and Russia [47]. Although CTX-M has only been around since the 2000s, it has dominated the TEM and SHV variants. The increase in CTX-M variants is likely due to selection pressure from the uncontrolled use of cephalosporins and more effective mobilization of CTX-M genes by mobile genetic elements. This might be related to the low fitness cost of expressing CTX-M enzymes in the host bacteria [48].

Studies have shown that farmers are more likely to be infected with ESBL-producing bacteria than people who do not have direct contact with dairy cattle. These events provide an overview of the spread of ESBL from dairy farms to the community [49]. Although the prevalence of ESBL-producing *K. pneumoniae* in wastewater from dairy farms in East Java is low, the results should be approached with caution considering that dairy farming in Indonesia is dominated by traditional breeders [50]. Dairy cattle farms in Indonesia generally dispose of waste into rivers without prior processing [51]. Rivers can be important reservoirs for the spread of resistant genes, especially ESBLs, to bacteria downstream [52]. The main cause of the increasing prevalence of MDRB to beta-lactam class antibiotics is the transfer of genes in plasmids, integrons, and transposons [28]. Transposons, gene insertion sequences, and integrons play an important role in disseminating ESBL genes in the same bacterial genome [53].

The presence of ESBL-producing bacteria results in a limited selection of drugs. Resistant bacteria increase mortality, morbidity, and medical costs. From 1980 to 1990, nosocomial infections were caused by *K. pneumoniae*, the main producer of ESBL bacteria, and not *E. coli* [53]. Resistant bacteria are produced due to the irrational and reckless use of antibiotics [54]. High concentrations of antibiotic residues in the environment support the proliferation of resistant bacteria. Free disposal of liquid waste into the environment is a serious problem for public health, especially in developing countries [55]. This problem requires strict policies and public awareness to prevent the increase of bacterial risk factors for MDR, especially ESBL-producing *K. pneumoniae*, in dairy farm wastewater, in East Java Province.

Conclusion

This study showed that 14.32% (49/342) of samples contained *K. pneumoniae*, with the prevalence of MDR at 12.67% (43/342) and of ESBL at 5.55% (19/342). Although the prevalence of ESBL-producing *K. pneumonia* in wastewater from dairy farms in East Java was low, caution should be exercised to prevent these from acting as a reservoir for ESBL.

Authors' Contributions

FNAEPD: Conceptualized and designed the study and writing-original draft. SMY, AW, RS: Interpreted the data. MHE: Conceptualization and design of the study, edited the final manuscript. HP and WT: Conceptualization and design of the study. ENU: Writing-original draft and revised the manuscript. MAAS: Performed laboratory procedures and collected samples. All authors have read, reviewed, and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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