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Multi-Drug Resistant (MDR) Detection in *Klebsiella Pneumoniae* in Canary Birds (*Serinus canaria*) Imported from Malaysia

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Abstract

Antimicrobial resistance (AMR) is a top priority for the WHO and the EU Commission, considering it a top 10 threat to global public health. This study provides an overview of the potential spread of *Klebsiella pneumoniae*, which exhibits multi-drug resistance (MDR), as a reservoir for the spread of resistance genes in the community. Samples were incubated on an MCA medium for isolation, followed by a Gram stain test and an IMViC test for further identification. The Kirby-Bauer diffusion test was used to determine antibiotic sensitivity. Based on the morphological characterization of the cultures, Gram stain results, and biochemical tests, it was found that, of the 150 samples isolated, 12 (8%) were positive for *K. pneumoniae*; 91.66% (11/12) of the isolates showed the highest level of resistance to amoxicillin, 83.33% (10/12) to tetracycline, 66.66% (8/12) to ciprofloxacin, and 66.66% (8/12) to trimethoprim-sulfamethoxazole. As many as 83.33% (10/12) were identified as MDR as they showed resistance to three to four types of antibiotics. Judicious use of antibiotics, including proper selection of antibiotics and monitoring of their usage patterns, is key to maintaining treatment effectiveness. Joint efforts from various parties are needed to optimize the use of antibiotics and minimize the risk of bacterial resistance.

Keywords

Bacteria *Klebsiella pneumoniae*, Communicable disease, Infectious diseases, multi-drug resistance, public health concern.

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Introduction

The domestic cat, scientifically known as *Felis catus*, is a beloved member of households worldwide, making it one of the most popular pets. Cats come in various forms, including purebred and local cats that result from crossbreeding or living in the wild (De Oliveira et al., 2001). From an economic standpoint, cat breeding can be a profitable venture due to the remarkable reproductive potential of these animals.

Female cats, in particular, exhibit impressive reproductive capabilities, often giving birth to litters of one to six kittens in a single reproductive cycle. However, alongside this fertility, cats are susceptible to various reproductive disorders and diseases (Thomson and Britt, 2022). These conditions can not only affect the health of the cats but also their ability to produce healthy offspring, which is critical for breeders and pet owners. Reproductive disorders in cats encompass a range of issues, including infections in the reproductive tract, reproductive tumors, hormonal imbalances, and other disorders (Thomson and Britt, 2022). Among these conditions, fetal mummification is a peculiar and potentially life-threatening phenomenon.

Fetal mummification occurs when a fetus in the uterus dies without becoming contaminated by microorganisms. In such cases, the dead fetus is gradually absorbed by the mother's uterus, leading to a dry and hardened mummified state. Several factors can trigger fetal mummification, including umbilical cord entanglement, uterine torsion, umbilical cord constriction, and genetic abnormalities. This condition manifests through various symptoms, such as the presence of a rigid fetus, anestrus, persistent straining, and anorexia (Affandhy et al., 2007).

Diagnosing fetal mummification relies on a comprehensive approach, encompassing indications, reproductive history, clinical symptoms, and physical examinations, supported by various diagnostic tools. These investigative methods include X-ray radiography, ultrasonography (USG), and blood hematology tests (Santana et al., 2019).

Ultrasound, in particular, is an invaluable diagnostic aid for fetal mummification, as it can reveal vital signs of lifelessness, such as the absence of fetal movement and a detectable heartbeat. Ultrasound is a versatile tool for detecting various pregnancy abnormalities (Noviana et al., 2008). X-ray radiography can provide further insights, revealing the presence of ossified and drying mummified fetuses. Additionally, blood hematology tests serve as essential supportive tools by indicating changes in blood composition related to potential infections or inflammation caused by fetal mummification.

Treating cases of fetal mummification in cats involves two main therapeutic options: pharmacological intervention through the injection of PGF2α to induce uterine contractions and surgical procedures. PGF2α injection is aimed at stimulating uterine contractions to facilitate the expulsion of the mummified fetus naturally. Surgical interventions are usually performed through ovariohysterectomy (OH), a procedure involving the removal of the ovaries, uterine horns, and uterine corpus from the abdominal cavity.

In cases of fetal mummification, the choice of treatment method is critical, as it significantly affects the cat's overall health, reproductive prospects, and well-being. Understanding the implications of these

treatment options is crucial for both veterinarians and cat owners. The ovariohysterectomy method, commonly known as OH, is a particularly important aspect of treating fetal mummification. It can prevent the recurrence of this condition and safeguard the cat from other reproductive diseases such as pyometra, metritis, and endometritis. Therefore, gaining an in-depth understanding of this method is essential for all those involved in feline care and breeding.

This research seeks to shed light on the significance of the ovariohysterectomy (OH) technique as a valuable tool in managing cases of fetal mummification in cats. By providing a comprehensive explanation of the OH method, this study aims to equip future veterinarians with the knowledge and skills necessary to effectively apply this technique in their professional practice. Moreover, this research is instrumental in promoting a deeper understanding of the benefits of ovariohysterectomy as a therapeutic approach for cases of fetal mummification in cats. By offering detailed insights into the procedure, its outcomes, and potential complications, this study empowers veterinarians to make informed decisions when treating affected cats.

Furthermore, the urgency of this research lies in its critical relevance to the welfare and reproductive health of cats. Fetal mummification is a severe condition that necessitates precise and timely intervention. By enhancing the understanding of ovariohysterectomy and its role in managing this condition, veterinarians can provide better care for afflicted cats, reduce the risk of complications, and improve the animals' overall quality of life. In conclusion, this study aims to address the critical need for comprehensive knowledge about the OH technique in the context of managing fetal mummification in cats. By offering a detailed exploration of this method, its implications, and its benefits, this research contributes to a more complete understanding of the management of fetal mummification in cats. Antimicrobial resistance (AMR) has received priority attention from the World Health Organization (WHO) and the EU Commission as one of the top 10 threats to global public health (Hamad *et al*., 2019; Murray *et al*., 2019; WHO, 2023). *Klebsiella pneumoniae* bacteria are classified as sensitive, intermediate, and resistant to many classes of antibiotics (MDR) (Sequeira *et al*., 2020). *K. pneumoniae* can cause infections of the respiratory system, skin, soft tissue, urinary tract, and septicemia (Navon-Venezia *et al*., 2017; Pham *et al*., 2023; Shree *et al*., 2024). Antibiotic resistance is an important issue in animal and human health, and multi-drug resistance (MDR) refers to a state where bacteria become resistant to three or more different types of antibiotics (Gomez et al., 2021). Pet birds are considered a source of multi-drug resistance (Diren Sigirci *et al*., 2020; Aklilu *et al*., 2022).

Enterobacteria are a group of Gram-negative bacteria (GNB) that have a high degree of pathogenicity and are prone to increased resistance to antimicrobials (AMR). One of the significant species in this group is *Klebsiella pneumoniae* (Paczosa *et al*., 2016). *K. pneumoniae* is frequently involved in human infections and caused the deaths of 600,000 people worldwide in 2019, and, if left unaddressed, MDR bacteria could cause up to 10 million deaths annually by 2050 (O'Neill,

2016; Murray *et al*., 2022). In the context of the pandemic caused by SARS-CoV-2, *K. pneumoniae* is known to cause approximately 55% of nosocomial respiratory co-infections in COVID-19 patients and increase the risk of death associated with infectious diseases by 2.5-fold (García-Meniño *et al*., 2021; Shree *et al*., 2024). Given the spread of MDR-*K. pneumoniae* infections, studies that pay attention to the transmission risks associated with the prevalence of this pathogen are of great importance. *K. pneumoniae* is also considered an innate pathogen, along with extraintestinal Escherichia coli (Riley, 2020).

Multi-drug resistance is caused by *K. pneumoniae* carrying resistance genes, and plasmid-borne quinolone resistance is spread in the environment, animals, and food samples (Zhang *et al*., 2018; Wareth and Neubauer, 2021; Aslam *et al*., 2022; Wu *et al*., 2022). Several publications have reported it as a zoonotic disease, and it has been included in the European Center for Disease Prevention and Control's list as another potential zoonotic agent (Santaniello *et al*., 2020; Hu *et al*., 2021). Key virulence factors of *K. pneumoniae*, such as hypermucoviscous phenotype (HMV), iron-binding siderophore production, lipopolysaccharide production, and capsule hyperproduction, are an important focus for evaluating the potential risk of zoonotic transmission (González-Ferrer *et al*., 2021).

Research on MDR *K. pneumoniae* and the high number of imported birds prompted the need for a study investigating the presence of *K. pneumoniae* in cross-border imported canaries. This study is expected to provide an overview of the potential spread of MDR-producing *K. pneumoniae* through the flow of imported canaries, which could become a reservoir for the spread of resistance genes in the community. Through a deeper understanding of *K. pneumoniae*, it is possible to estimate the risk of zoonotic MDR transmission.

Materials and Methods

Sample collection

The research was conducted from February 2023 to April 2023 at the Canary Importer in Malang Regency. A total of 150 imported canary feces samples that met the criteria of fresh feces were taken using a sterile swab stick (Oxoid, Bangistoke, UK) aseptically then inserted into a tube containing 2% Buffer Peptone Water (BPW) (Merck) and labeled. Samples were then stored in a cool box and brought to the laboratory for further analysis.

Isolation and identification

Isolation and identification were observed in *K. pneumoniae* isolates during the three months involved in this study. *K. pneumoniae* isolates were identified using standard biochemical tests. Canary fecal swab samples (n = 150) were grown on McConkey Agar (MCA) medium by the streak method and incubated at 37°C for 18-24 hours. One colony with typical *K. pneumonia* morphology was selected. It was then identified using conventional methods (Silva-Bea *et al*., 2024).

Sensitivity test

The antibiotic sensitivity test using the Kirby-Baurer method, and the disk diffusion test produce qualitative categories with sensitive, intermediate, and resistant assessments (Indana *et al*., 2020). Cultured bacteria were obtained from colonies that grew on MCA media and then planted in test tubes containing 8 ml of physiological NaCl solution. Homogenization was carried out using a vortex until the turbidity reached the same level as the McFarland 0.5 standard. After that, 0.2 ml of isolate was taken and applied evenly to the

entire surface of Mueller Hinton Agar (MHA) media. Bacteria were allowed to stick to the media for 15 minutes, then antibiotic disks were placed on MHA media. The disc was slightly pressed on the surface of the agar, and then the bacterial culture incubated at 37°C for 24 hours.

Results

Isolation and identification

The results of morphological culture, Gram staining, and biochemical testing showed 8% (12/150) of presentive *K. pneumonia* (Table 1). The appearance of pink and mucoid bacterial colonies on MCA media indicated the success of the morphological culture of *K. pneumonia* (Figure 1). It has a short rod-shaped morphology (Figure 2), has a capsule, and does not form spores. *K. pneumonia* bacteria were identified through a series of biochemical tests, including the TSIA test which showed positive results indicated by a color change from red to yellow on the acid slant and acid butt sections. The test results also showed positive gas production marked by the lifting of TSIA media and negative H_2S, marked by the formation of no black color on the media. The IMViC test (Figure 3), in the Sulfide Indole Motility (SIM) test, showed negative results for sulfide production, namely the formation of no black color in the media and negative indole production marked by no red ring forming on the surface of the media after being tested with Kovac's reagent. The motility test also showed negative results marked by the absence of blurriness in the osseous puncture area. The Methyl Red-Voges Proskauer (MR-VP) test gave a negative result marked by no color change when tested with MR reagent, while the Voges-Proskauer test gave a positive result marked by a change in color to red when tested with VP reagent. The Simmons Citrate Agar (SCA) test showed a positive result marked by a color change in the media from green to blue, indicating that the bacteria were able to utilize citrate as a carbon source. The sugar test (sucrose, lactose, glucose, and mannitol) gave a positive result marked by a color change in the media from red to yellow and gas formation in the Durham tube.

Table 1. IMViC test

Test	MacConkey Agar	Indole	Methyl Red	Voges Proskauer	Citrate	TSIA
Total	12	12	12	12	12	12

Figure *1*. *Klebsiella Pneumoniae* colonies in MacConkey Agar.

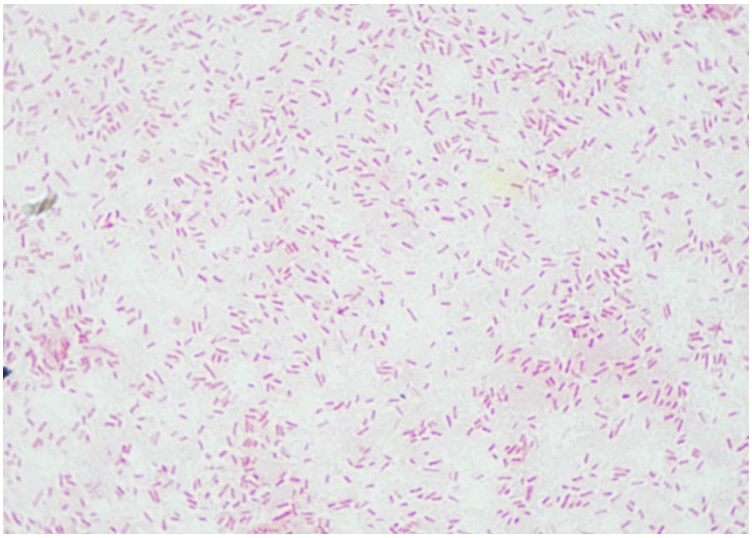

Figure 2. Gram-stained of *Klebsiella Pneumoniae* colonies under the microscope.

Figure 3: IMViC test results indicate positive for *Klebsiella Pneumoniae*.

The results of the study are similar to the findings of Suarjana *et al*. (2021), showing short rod-shaped bacteria with Gram-negative properties after Gram staining, which is caused by lipid-coated cell walls. The catalase test showed positive results with the formation of gas bubbles, while the oxidase test gave negative results. The TSIA test showed positive results in sucrose, glucose, and lactose fermentation, as well as gas production, while the IMViC and MR-VP tests gave negative results for some parameters, and the Voges-Proskauer and SCA tests gave positive results. The sugar test showed positive results with a color change from red to yellow on the media and gas formation, and according to the research of Putra *et al*. (2023), *Klebsiella sp*. isolates show Gram-negative characteristics and a short rod shape in Gram staining. Biochemical tests showed that *Klebsiella sp*. fermented sucrose, glucose, and lactose with gas production on TSIA media. The SIM test showed negative results for sulfide and indole, and negative results for motility. MR-VP test results showed positive for Voges-Proskauer and negative for Methyl Red. The sugar test also showed positive results.

Physical examination showed that the canaries looked dehydrated and weak, and showed symptoms of diarrhea and lethargy. The clinical symptoms found in canaries infected with *K. pneumoniae* are supported by the results of Nakhaee *et al*. (2022), who stated that clinical symptoms of *K. pneumoniae* infection in canaries included anorexia, lethargy, diarrhea, and mortality of about 30% within two days. On physical examination, the birds appeared very weak, deformed, and dehydrated with matted feathers, and wet discolored feces around the cloaca.

Antibiotic resistance

The results of the antibiotic resistance analysis against *K. pneumoniae* isolates showed that there was a zone of inhibition formed after

the media was incubated with antibiotics for 24 hours at 37°C, then measured using a caliper. Furthermore, the inhibition zone measurement results were compared with the standards given by CLSI (2020) (Table 2). Of the 150 isolates tested, 10 of them showed resistance to three to four different types of antibiotics. The analysis showed that 91.66% (11/12) of *K. pneumoniae* isolates showed the highest level of resistance to amoxicillin, while 83.33% (10/12) of isolates showed resistance to tetracycline. In addition, 66.66% (8/12) isolates showed resistance to ciprofloxacin and trimethoprim-sulfamethoxazole. However, no isolates were found that showed resistance to ceftazidime, as listed in Table 3. The antibiotic resistance profile based on the results of the *K. pneumoniae* resistance test showed that of the 12 positive isolates of *K. pneumoniae*, 10 isolates (83.33%) were confirmed to be MDR because they were resistant to three to four classes of antibiotics (Figure 4). The antibiotic resistance pattern was as follows: AML- CIP- SXT, TE-AML-CIP, TE-AML-SXT, and TE-AML-SXT-CIP, as listed in Table 4.

Table 2. Interpretation of inhibition zone measurements based on the provisions of the Clinical and Laboratory Standard Institute (CLSI, 2020).

Antibiotics	Disk Content	Resistant ≤ mm	Intermediate	Sensitive ≥ mm
Ceftazidime	30 µg	17	18-20	21
Amoxicillin	10 µg	13	14-17	18
Ciprofloxacin	5 µg	21	22-25	26
Tetracycline	30 µg	11	12-14	15
Trimethoprim sulfamethoxazole	23.75 µg	10	11-15	16

Table 3. Resistance Test

Antibiotics	Resistant	Intermediate	Sensitive
CAZ	-	3	9
AML	11	1	-
CIP	8	1	3
TE	10	-	2
SXT	8	-	4

Notes: CAZ = Ceftazidime, AML = Amoxicillin, CIP = Ciprofloxacin, TE = Tetracycline, SXT = Trimethoprim sulfamethoxazole.

Table 4. Resistance Pattern

Resistance Pattern	Total
TE-AML-SXT-CIP	5
TE-AML-SXT	2
TE-AML-CIP	2
AML-CIP-SXT	1
MDR	10

Notes: CAZ = Ceftazidime, AML = Amoxicillin, CIP = Ciprofloxacin, TE = Tetracycline, SXT = Trimethoprim sulfamethoxazole.

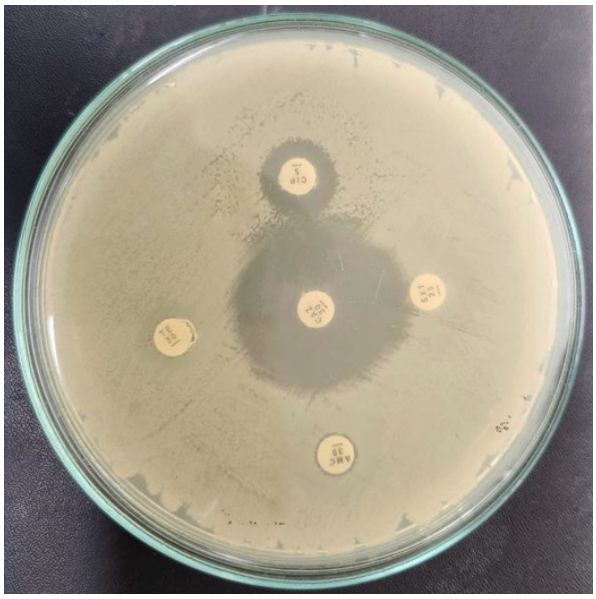

Figure 4: Analysis the susceptibility to antibiotics of *Klebsiella Pneumoniae* isolate cultured on Mueller Hinton Agar.

Antibiotic resistance is one of the serious challenges facing global public health. Organisms resistant to antibiotics can spread rapidly, bringing risk to populations with infectious diseases that are difficult to cure and treat (Aminul *et al*., 2021). MDR *K. pneumoniae* has become a major concern in healthcare systems due to its ability to limit treatment options (Huy, 2024). Biofilm formation is a major virulence factor that contributes to the persistence of infections by interfering with antimicrobial effectiveness and creating an environment that favors the horizontal transmission of antibiotic-resistance genes (González-Ferrer *et al*., 2021; Guerra *et al*., 2022). Bacteria that form biofilms will show resistance to various antimicrobials (Ramos-Vivas *et al*., 2019). The mechanism of MDR through the K. pneumoniae genome involves the acceptance of antibiotic resistance genes through plasmids with transferable genetic elements, leading to the emergence of strains resistant to various antibiotics (Navon-Venezia *et al*., 2017; Zhang *et al*., 2018).

Of the 12 *K. pneumoniae* isolates, 91.66% (11/12) showed the highest level of resistance to amoxicillin. Amoxicillin is a broad-spectrum β-lactam antibiotic that is bactericidal, and effective against Gram-positive and Gram-negative bacteria (Maida and Kinanti, 2019). ESBL-producing *K. pneumoniae* can cause resistance to the penicillin group, namely amoxicillin (Ahmad *et al*., 2022). Resistance to amoxicillin is caused by various types of resistance genes such as blaTEMP, blaCTX, blaSHV, blaOKA, blaCMY, blaDHA, blaPER, blaVEB, blaIMP, blaTLA, blaKPC, blaVIM, blaNDM, blaKLUC-5, blaSFO, blaGES, blaMOX, and blaRubah (Wong *et al*., 2015; Chung, 2016; Lee *et al*., 2016). Bacteria that have β-lactamase enzymes can cause resistance to ß-lactam antibiotics, especially in Gram-negative bacteria. This enzyme can inactivate the effect of antibiotics by breaking the ß-lactam ring (Pratiwi, 2017). This statement is reinforced by Galani *et al*. (2018) who stated that the resistance of *K. pneumoniae* bacteria involves the mechanism of carbapenemase production.

Carbapenemase is a β-lactamase enzyme that uses carbapenem as a hydrolysis substrate (Santoso and Rostinawati, 2022).

There were 83.33% (10/12) *K. pneumoniae* isolates that showed resistance to tetracycline. Tetracycline is a type of antibiotic that is bacteriostatic (Simanjuntak *et al*., 2022). The mechanism of action of tetracycline mainly occurs by inhibiting protein synthesis in the 70s and 80s subunits of the ribosome. Resistance to tetracycline is due to the presence of resistance genes, which are located extra-chromosomally and can replicate and synthesize proteins required for plasmids (Agustanty and Andre, 2022). Genetic factors that contribute to resistance to tetracycline include Ade-ABC efflux pumps, KpgABC, Oqx-AB, Tet(A) mutants, and ribosomal proteins. These antibiotic-resistance genes are localized on chromosomes and have changes in cell permeability and ribosome targeting. In addition, structural mutations in the S3 protein can also lead to resistance to tetracycline. Studies have also suggested that mutations in the RpsJ gene can induce resistance to tetracycline without the involvement of efflux pumps, as reported by Li *et al*. (2023).

There were 66.66% (8/12) isolates that showed antibiotic resistance to trimethoprim-sulfamethoxazole, and ciprofloxacin. The ESBL resistance gene contained in the plasmid also carries resistance to another class of drugs, namely trimethoprim-sulfamethoxazole (Ahmad *et al*., 2022). Bacteria develop resistance to trimethoprim-sulfamethoxazole through five main mechanisms: naturally insensitive target enzymes, mutations or recombination changes in target enzymes, permeability barriers, and drain pumps, regulatory changes in target enzymes, and resistance acquired from drug-resistant target enzymes (Yekani *et al*., 2018). Meanwhile, resistance to ciprofloxacin arises from treatment failure caused by microbial resistance to normally effective broad-spectrum antibiotics. These treatment failures and intractable infections can increase mortality rates. The mechanisms of ciprofloxacin resistance found include changes in target enzymes, changes in drug permeability, and plasmid-mediated quinolone resistance (Aslam *et al*., 2018).

The results of research by Karimi *et al*. (2021) revealed that more than 90% of isolates were resistant to cefotaxime and piperacillin-tazobactam. In addition, more than 70% of isolates were resistant to ceftazidime and trimethoprim-sulfamethoxazole. According to research by Yekai *et al*. (2019), based on disc diffusion and agar dilution tests, the highest frequency of resistance was found to trimethoprim-sulfamethoxazole (96.8%), ampicillin (86.3%), cefazolin (79.4%), trimethoprim (78.5%), and nalidixic acid (68.5%). Low-level resistance was observed to fosfomycin (2.7%) and carbapenems (3.2%). According to the agar dilution test, the resistance rates to ciprofloxacin, nalidixic acid, and levofloxacin were 66.2%, 68.9%, and 58.5%, respectively. The resistance rates to tobramycin, kanamycin, gentamicin, and amikacin were 47.9%, 39.3%, 27.8%, and 5.5%, respectively. The highest frequency of resistance was found against trimethoprim-sulfamethoxazole (69.8%), sulfamethoxazole (88.1%), and trimethoprim (78.5%).

Inappropriate use of antibiotics can result in increased bacterial resistance, which emphasizes the need and importance of implementing rational use of antibiotics (Zhang *et al*., 2018). Bacterial resistance to antibiotics can increase through a selection pressure mechanism, in which resistant bacteria can

multiply rapidly through the duplication process, which usually takes about 20-30 minutes (Soleha *et al.*, 2019). In general, empirical antibiotics are given in the early stages of treatment. Therefore, regular monitoring of bacterial sensitivity patterns needs to be done to choose the right antibiotic. The selection of appropriate antibiotics is not only important to ensure the success of therapy, but also to prevent the emergence of cases of immunity to multi-drug resistance (MDR) (Setyowati and Silviani, 2020).

Judicious use of antibiotics is an approach that emphasizes the selection of narrow-spectrum antibiotics that are appropriate for the right indication, an adequate dose, and an appropriate duration of use. Antibiotic resistance is a global issue of concern, with its prevalence continuing to increase globally. To face this challenge, concerted efforts are needed from various parties. One strategy is to monitor the trade of canaries, which can be a source of resistance gene spread in various countries. In addition, it is also important to encourage the rational use of antibiotics, as well as monitor and evaluate the pattern of antibiotic use and make appropriate interventions to optimize the use of antibiotics.

Conclusion

The identification results showed that *K. pneumoniae* was detected in 8% (12/150). Antibiotic sensitivity tests confirmed that 10 *K. pneumoniae* isolates could be categorized as MDR because they showed resistance to three to four types of antibiotics, with the highest level of resistance to amoxicillin. Antibiotic resistance, particularly in *K. pneumoniae*, is a serious challenge to global public health. The importance of monitoring trade in animals, such as canaries, as a potential source of the cross-border spread of antibiotic resistance was also emphasized. In the face of this problem, judicious use of antibiotics, including appropriate selection of antibiotics and monitoring of usage patterns, is key to maintaining treatment effectiveness. Joint efforts from various parties are needed to optimize the use of antibiotics and minimize the risk of bacterial resistance.

Approval of Ethical Commission

All tests were carried out without causing harm or sacrificing any live animals. Hence, ethical clearance for animal experiments was considered unnecessary. Field investigations were conducted with official approval from the Quarantine Agency in Indonesia, guaranteeing that no animals were harmed throughout the study. Furthermore, no human participants were involved in this research.

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Author's Contribution

TE, MHF, and M'AK: Conceptualized, designed, and coordinated the study. TE, FK, and DAR: Developed data collection tools and supervised sampling, field data collection, laboratory procedures, and data entry. TEs and IIs: Conducted validation, supervision, and formal analysis. MHF and M'AK: Performed statistical analysis, interpreted statistical results

and contributed to drafting the manuscript. All authors have read, reviewed, and approved the final manuscript.

Conflict of Interest

The authors declare no relevant conflicts of interest related to this research, whether financial, personal, or professional.

Data Availability Statement

The data used in this study are the result of our research and have been included in this manuscript, including tables, graphs, and appendices

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