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Molecular identification of *Klebsiella pneumoniae* that produces extended spectrum β-lactamase (ESBL) in canaries (*Serinus canaria*) imported from Malaysia

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ABSTRACT

Klebsiella pneumoniae is an opportunistic Gram-negative bacterium known to cause serious infections in humans and animals and has the ability to develop resistance to various antibiotics. One of the most concerning resistance mechanisms is the production of Extended Spectrum Beta-Lactamase (ESBL) enzymes, which can hydrolyze the latest generation of β-lactam antibiotics. This study aimed to identify the presence of ESBL-encoding genes, specifically bla_{TEM} and bla_{SHV}, in *K. pneumoniae* isolates obtained from imported canaries (*Serinus canaria*). A total of 150 fresh fecal samples were collected from imported canaries in Malang Regency and analyzed using conventional microbiological methods. Identification was carried out through colony morphology characterization, Gram staining, and biochemical tests (IMViC, SIM, TSIA). Isolates identified as *K. pneumoniae* were then tested for sensitivity to five types of antibiotics using the disk diffusion method and continued with molecular detection of ESBL genes using PCR techniques. The results showed that 12 samples (8%) were positive for *K. pneumoniae*, and of these, 10 isolates (83.3%) showed resistance to ≥3 classes of antibiotics, categorized as multidrug-resistant (MDR). Molecular detection revealed that 6 isolates carried the bla_{TEM} gene and 1 isolate carried the bla_{SHV} gene. These findings indicate that imported canaries have the potential to be a reservoir of ESBL-producing *K. pneumoniae* and may contribute to the spread of antimicrobial resistance across species. Surveillance and early detection efforts are needed within the context of a One Health approach to prevent the risk of zoonoses and the spread of resistance in the global environment.

Introduction

The canary (*Serinus canaria*) has long been a popular songbird among Indonesians due to its melodious song and attractive plumage (Akrom*et al*., 2020). This bird's popularity extends beyond Indonesia and is widely recognized in various countries. Canaries are easily recognized by their distinctive morphological characteristics, such as their bright, clean plumage, small body size, thick, short beak, and translucent white feet with three toes pointing forward and one backward (Hidayat *et al*., 2015; Yunadia *et al*., 2024). *Klebsiella pneumoniae* is an encapsulated Gram-negative bacterium known as an opportunistic pathogen that causes various serious infections, including pneumonia, urinary tract infections, bacteremia, meningitis, and liver abscesses (Riwu *et al*., 2022).

Various strains of *K. pneumoniae* have become a global clinical threat due to their hypervirulence and resistance to multiple antibiotic classes (Velazquez-Meza *et al*., 2022). multidrug-resistant (MDR) strains are isolates that exhibit resistance to three or more antibiotic classes (Exner *et al*., 2017). The increasing prevalence of MDR isolates and Extended-Spectrum β-Lactamase (ESBL)-producing isolates has been widely reported in various countries, including in livestock and pets (Ansharieta *et al*., 2021). ESBLs are enzymes capable of hydrolyzing and inactivating various β-lactam antibiotics, including third-generation cephalosporins and monobactams (Shrestha *et al*., 2017). Furthermore, ESBL-producing bacteria often exhibit cross-resistance to other antibiotic classes such as aminoglycosides, quinolones, and trimethoprim-sulfamethoxazole (Abayneh *et al*., 2018). The most common ESBL-encoding genes are bla_{TEM} and bla_{SHV}. The bla_{TEM} gene is typically found on plasmids, while bla_{SHV} can be encoded by a resistance gene located on the chromosome (Permatasari *et al*., 2020). Infection caused by ESBL-producing strains is associated with an increased risk of mortality compared to infection by non-ESBL strains (Ling *et al*., 2021). In particular, mortality is higher in patients infected with ESBL-producing *K. pneumoniae* (Sianipar *et al*., 2019).

Research conducted by Yang *et al*. (2020) showed that *K. pneumoniae* has a high level of resistance to various classes of antibiotics, including penicillin, third-generation cephalosporins, monobactams, macrolides, tetracyclines, fluoroquinolones, trimethoprim, and chloramphenicol. The bla_{SHV} gene was first identified as an ESBL type in a clinical isolate of *Klebsiella ozaenae* in Germany and was later known as SHV-2. This gene is horizontally transferable, and variants such as SHV-2, SHV-5, and SHV-12 are commonly found in bacteria from the Enterobacteriaceae family (Liakopoulos *et al*., 2016). This is supported by research by Dirar *et al*. (2020), which reported the presence of the bla_{TEM}, bla_{CTX-M}, and bla_{SHV} genes in 34, 31, and 26.1% of *K. pneumoniae* isolates, respectively, indicating that ESBL-producing isolates often carry more than one resistance gene. As an opportunistic pathogen, *K. pneumoniae* not only causes infections in humans and animals but is also found as a common contaminant in

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meat products (Hartantyo *et al*., 2020). The increasing prevalence of MDR strains with high virulence levels poses a serious threat to food safety as well as animal and human health (Yunita *et al*., 2020; De Koster *et al*., 2022).

Although *K. pneumoniae* has been widely reported as an agent of nosocomial and zoonotic infections, studies on the presence of ESBL-producing strains in imported birds are still very limited. Given the high volume of international trade in canaries, especially from countries such as Malaysia, adequate surveillance is needed to detect the presence of ESBL-producing *K. pneumoniae* in this species. Therefore, this study aims to identify antibiotic-resistant *K. pneumoniae* isolates and detect the presence of ESBL-encoding genes, namely bla_{TEM} and bla_{SHV}, in imported canaries. This study aimed to evaluate the potential of canaries as a reservoir of antimicrobial resistance and provide a preliminary overview of the risk of transboundary spread through global pet trade.

Materials and methods

Research design

This study was conducted from February to April 2023. Imported canary (*Serinus canaria*) fecal samples were obtained from a canary importer located in Malang Regency, East Java. A total of 150 fresh fecal samples were collected aseptically using sterile swab sticks (Oxoid, Basingstoke, UK), then placed in tubes containing 2% Buffer Peptone Water (BPW) and labeled with identification. All samples were stored in a cooler box and immediately sent to the laboratory for further analysis.

Isolation and identification

The obtained fecal swabs (n = 150) were inoculated into McConkey Agar (MCA) media using the streaking method, then incubated at 37°C for 18–24 hours. Colonies with typical *K. pneumoniae* morphology were selected and further identified using conventional methods based on biochemical characteristics (Silva-Bea *et al*., 2024).

Antibiotic sensitivity test

Antibiotic sensitivity testing was performed using the Kirby-Bauer disk diffusion method on Mueller Hinton Agar (MHA) media according to standard procedures. The bacterial suspension was prepared to a turbidity level equivalent to the McFarland standard of 0.5, then 0.2 mL was spread evenly over the entire surface of the MHA media. After standing for 15 minutes, the antibiotic disc (Ceftazidime, amoxicillin, ciprofloxacin, tetracycline, and trimethoprim-sulfamethoxazole) was placed on the media surface and gently pressed. Incubation was carried out at 37°C for 24 hours. Results were observed based on the inhibition zone formed and classified as sensitive, intermediate, or resistant (Prayudi *et al*., 2023).

ESBL gene detection by PCR

K. pneumoniae isolates from MCA media were then re-inoculated into MHA media and incubated at 37°C for 24 hours. One colony of the isolate was transferred into a microcentrifuge tube (Eppendorf safe-lock tube) containing 300 µL of TE buffer (10 mM Tris-HCl, pH 8; 10 mM EDTA). The suspension was vortexed, and DNA extraction was performed using a modified boiling lysis method using thermoStat™ at 98°C for 10 minutes. After that, the sample was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was stored at −20°C until used for PCR (Riwu *et al*., 2024).

ESBL gene amplification (bla_{SHV} and bla_{TEM})

PCR reactions were performed in a total volume of 20 µL, consisting of 12.5 µL GoTaq® Green Master Mix (Promega), 1 µL each of the forward and reverse primers for the bla_{SHV} or bla_{TEM} gene, 0.5 µL of DNase-free water, and 5 µL of template DNA. The reaction mixture was transferred to a PCR tube and amplified using a thermal cycler with a modified cycle. The amplification process was concluded with a final extension step at 72°C for 2 minutes. The same procedure was applied for the amplification of the bla_{TEM} gene (Kurniawan *et al*., 2025).

Results

A total of 150 imported canary (*Serinus canaria*) feces samples were collected from Malang Regency. The samples were stored in Buffer Peptone Water (BPW) media and transported in a cool box to the laboratory for bacterial isolation and identification. Planting the samples on McConkey Agar (MCA) media produced presumptive colonies of *K. pneumoniae* that showed a typical morphology of round, convex, mucoid, and pink colonies. The appearance of the colonies on MCA media is presented in Figure 1. For further confirmation, Gram staining was performed on the isolates, and microscopic observations at 1000x magnification showed short, pink rod morphology indicating Gram-negative nature, as shown in Figure 2.

Figure 1. Presumptive colonies of *K. pneumoniae* on McConkey Agar (MCA) medium exhibit a round, mucoid, and pink morphology. This colony color indicates lactose fermentation, while the slimy surface indicates the presence of a polysaccharide capsule, a characteristic of *K. pneumoniae*.

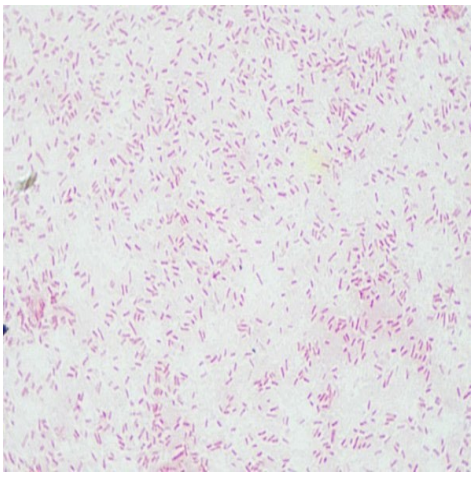

Figure 2. Gram staining of a presumptive isolate of *K. pneumoniae* at 1000x magnification. The bacteria appear short rod-shaped and pink, indicating that they are Gram-negative. These morphological characteristics are consistent with the microscopic characteristics of *K. pneumoniae*.

Presumptive colonies of *K. pneumoniae* grown on McConkey Agar (MCA) showed a typical morphology of round, convex, pink colonies with a mucoid surface. The pink color of the colonies indicates that the bacteria are able to ferment lactose, while the slimy texture reflects the production of a polysaccharide capsule, which is an important virulence

factor of *K. pneumoniae*. Initial identification was continued with Gram staining to observe the cell wall type and morphology of the bacteria. Microscopic observations at 1000x magnification showed that the isolate had a short rod shape and a pink color, indicating Gram-negative nature. This morphology is consistent with the characteristics of *K. pneumoniae* as a member of the Enterobacteriaceae family, and supports the initial identification results based on the appearance of colonies on selective media. To strengthen the identification results, the presumptive isolate of *K. pneumoniae* was further tested using a series of biochemical tests, including IMViC and Triple Sugar Iron Agar (TSIA), to obtain further information regarding the physiological characteristics and metabolic activity of the isolate. The results of the biochemical tests of the *K. pneumoniae* isolate are presented in Figure 3.

Figure 3. Biochemical test results of *K. pneumoniae* isolates using IMViC and TSIA media. From left to right: (1) Triple Sugar Iron Agar (TSIA); (2) SIM (Sulfur-Indole-Motility); (3) Methyl Red (MR); (4) Voges-Proskauer (VP); (5) Simmons Citrate Agar (SCA).

Confirmation of the identification of *K. pneumoniae* isolates was carried out through a series of biochemical tests including Triple Sugar Iron Agar (TSIA), Sulfide-Indole-Motility (SIM), Methyl Red (MR), Voges–Proskauer (VP), and Simmons Citrate Agar (SCA). The TSIA test showed that the isolate was able to ferment glucose, lactose, and sucrose, as evidenced by the color change of the entire medium to yellow. In addition, no black precipitate formed, indicating that the isolate did not produce hydrogen sulfide (H_2S). The presence of cracks and uplift of the medium indicated gas production during the fermentation process.

Furthermore, tests on SIM media showed that the isolate was non-motile, indicated by the absence of diffusion or spread of growth from the inoculation point on semi-solid media. The Indole test, also performed on SIM media, showed a negative result, indicated by the absence of a red ring after the addition of Kovac's reagent. The absence of black color in the media also confirmed the absence of H_2S production.

The Methyl Red test showed a negative result, indicated by the absence of a red color change after the addition of Methyl Red reagent, indicating that the isolate did not produce strong acid from glucose fermentation. In contrast, the Voges–Proskauer test showed a positive result, indicated by the formation of a pink–orange color after the addition of α-naphthol and KOH reagents and incubation for 30 minutes. This indicates the production of acetoin as a result of fermentation.

The test results on Simmons Citrate Agar showed a positive reaction, indicated by a change in the color of the medium from green to blue. This change indicates that the isolate is able to utilize citrate as its sole carbon source, which is one of the biochemical characteristics of *K. pneumoniae*. Overall, the biochemical profile obtained from a series of five supporting tests is consistent with the phenotypic characteristics of *K. pneumoniae*. Based on the results of these biochemical tests, 12 isolates (8%) presumptive *K. pneumoniae* were successfully identified from a total of 150 canary fecal samples collected in Malang Regency.

Of the 150 canary feces samples examined, 12 isolates (8%) showed pink colony growth on MacConkey Agar (MCA) media. This color indicates lactose fermentation ability, which is one of the characteristics of the *Klebsiella* genus. The isolates obtained were further characterized through Gram staining and a series of biochemical tests to confirm their identity as *K. pneumoniae*. The Gram staining results showed that all isolates were Gram-negative bacilli. Further biochemical tests showed that all isolates were negative for the indole test, hydrogen sulfide (H2S) production, and Methyl Red (MR) test; but positive for the Voges-Proskauer (VP) and citrate tests. In addition, all isolates were non-motile and showed glucose and lactose fermentation patterns without H2S production on Triple Sugar Iron Agar (TSIA) media. The obtained biochemical profiles were consistent with the phenotypic characteristics of *K. pneumoniae* reported in the microbiology literature. Thus, it can be concluded that 8% of the total samples were successfully identified as *K. pneumoniae* based on a combination of culture tests, microscopic morphology, and biochemical confirmation.

Isolates identified as *K. pneumoniae* were then tested for antibiotic sensitivity using the disc diffusion test. Testing was performed on five commonly used antibiotics: amoxicillin, ceftazidime, ciprofloxacin, trimethoprim-sulfamethoxazole, and tetracycline. The diameter of the inhibition zone formed around the antibiotic disc was measured using a vernier caliper. The complete results of the resistance patterns of *K. pneumoniae* isolates to the five antibiotics are presented in Table 1.

Table 1. Antibiotic resistance pattern of *K. pneumoniae* isolates (n = 12).

Antibiotic	Resistance	Intermediate	Sensitive
CAZ	–	3	9
AML	11	1	–
CIP	8	1	3
TE	10	–	2
SXT	8	–	4

Note: Ceftazidime (CAZ), amoxicillin (AML), ciprofloxacin (CIP), tetracycline (TE), trimethoprim-sulfamethoxazole (SXT)

Antibiotic sensitivity testing of 12 *K. pneumoniae* isolates revealed varying levels of resistance to five commonly used antibiotics in clinical practice. The test results showed that all nine isolates were sensitive to ceftazidime (CAZ), with three isolates showing intermediate sensitivity, and no isolates were found to be resistant to this antibiotic. In contrast, the highest resistance was recorded to amoxicillin (AML), with 11 of the 12 isolates (91.7%) showing resistance, and only one isolate showing intermediate sensitivity.

A fairly high level of resistance was also found to ciprofloxacin (CIP), with 8 isolates (66.7%) classified as resistant, one isolate intermediate, and three isolates sensitive. Tetracycline (TE) antibiotics also showed a significant resistance pattern, with 10 isolates (83.3%) resistant and only 2 isolates sensitive. Meanwhile, in testing for trimethoprim-sulfamethoxazole (SXT), 8 isolates (66.7%) were found to be resistant and 4 isolates (33.3%) were still sensitive.

Overall, the observed resistance patterns indicate that most *K. pneumoniae* isolates obtained from canary fecal samples exhibited high levels of resistance to β-lactam antibiotics, particularly amoxicillin, and tetracycline. Furthermore, moderate levels of resistance were also detected to ciprofloxacin and trimethoprim-sulfamethoxazole (SXT). These findings indicate a significant potential risk of antimicrobial resistance, emphasizing the importance of monitoring and rational use of antibiotics, particularly in non-productive animals that can act as reservoirs for resistant bacteria.

Assessment of multidrug resistance (MDR) status is based on the cri-

terion that isolates are categorized as MDR if they show resistance to three or more antibiotic groups. Based on the results of sensitivity tests on 12 *K. pneumoniae* isolates, 10 isolates (83.3%) met the MDR criteria because they showed resistance to three to four types of antibiotics tested. The resistance pattern of *K. pneumoniae* isolates classified as MDR is presented in Figure 4.

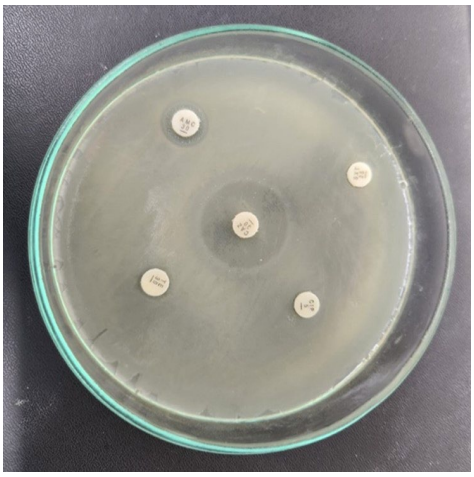

Figure 4. Antibiotic sensitivity test results for *K. pneumoniae* isolates using the disk diffusion method on Muller Hinton Agar (MHA) media. The inhibition zone formed around the antibiotic disk indicates the isolate's response to each antibiotic tested.

The results of antibiotic sensitivity testing on *K. pneumoniae* isolates using the disk diffusion method on MHA media. Five antibiotic discs are seen evenly placed on the surface of the media that has been inoculated with a suspension of test bacteria. The clear zone formed around each disc indicates the inhibition zone, which represents the effectiveness of the antibiotic against the tested isolate. The diameter of the inhibition zone was measured and analyzed to determine the level of sensitivity of the isolate to each antibiotic, based on the interpretative criteria established by the Clinical and Laboratory Standards Institute (CLSI). Variations in the size of the inhibition zone between discs reflect differences in the level of sensitivity or resistance of *K. pneumoniae* isolates to the tested antibiotics.

Antibiotic sensitivity testing using the disk diffusion method on 15 positive *K. pneumoniae* isolates against five types of antibiotics showed that 10 isolates (66.7%) met the criteria for multidrug resistance (MDR), namely resistance to three or more antibiotic groups. The resistance patterns of *K. pneumoniae* isolates classified as MDR are presented in detail in Table 2.

Table 2. Antibiotic resistance patterns in multidrug resistant (MDR) *K. pneumoniae* isolates.

Sample code	Resistance pattern
29	TE–AML–SXT–CIP
46	TE–AML–SXT–CIP
68	TE–AML–SXT–CIP
102	TE–AML-SXT–CIP
121	TE–AML-SXT–CIP
78	TE–AML–SXT
108	TE–AML–SXT
146	TE–AML–CIP
100	TE–AML–CIP
47	TE–CIP–SXT

Note: Ceftazidime (CAZ), amoxicillin (AML), ciprofloxacin (CIP), tetracycline (TE), trimethoprim-sulfamethoxazole (SXT)

Ten *K. pneumoniae* isolates identified as multidrug-resistant (MDR) showed varying resistance patterns to combinations of several antibiotic classes. By definition, MDR isolates are those that exhibit resistance to at least three antibiotic classes. In this study, five isolates (sample codes: 29, 46, 68, 102, and 121) showed resistance to four types of antibiotics: tetracycline (TE), amoxicillin (AML), trimethoprim–sulfamethoxazole (SXT), and ciprofloxacin (CIP), which is the most common resistance pattern among MDR isolates.

Three other isolates (sample codes: 78, 108, and 146) showed resistance to three types of antibiotics. Two isolates (78 and 108) had a TE–AML–SXT resistance pattern, while one isolate (146) showed a TE–AML–CIP pattern. Meanwhile, two other isolates (sample codes: 100 and 47) were also resistant to three antibiotics, with TE–AML–CIP and TE–CIP–SXT patterns, respectively. Overall, the combination of resistance to tetracycline and amoxicillin was the most dominant, accompanied by additional resistance to SXT or CIP.

This variation in resistance patterns indicates high antibiotic selection pressure in the environment and emphasizes the importance of monitoring antibiotic use in animals, including non-productive animals such as canaries, which have the potential to serve as reservoirs for resistant bacteria. Next, ten *K. pneumoniae* isolates confirmed to be MDR were analyzed for bla_{TEM} and bla_{SHV} gene detection. The results of bla_{TEM} gene detection in MDR isolates are shown in Figure 5, while the results of bla_{SHV} gene detection are shown in Figure 6.

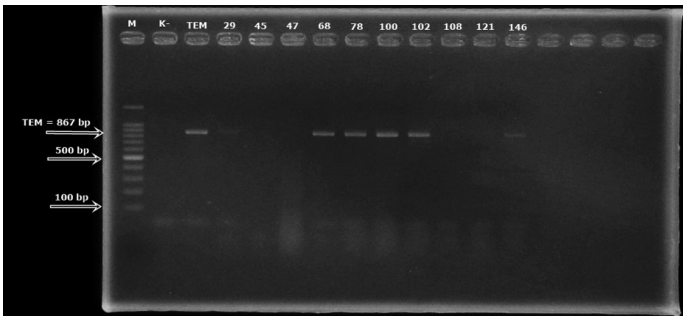

Figure 5. PCR electrophoresis results for the detection of the bla_{TEM} gene encoding the ESBL enzyme in *K. pneumoniae* isolates. M: DNA marker (100 bp ladder); K–: Negative control; TEM: Positive control of the bla_{TEM} gene.

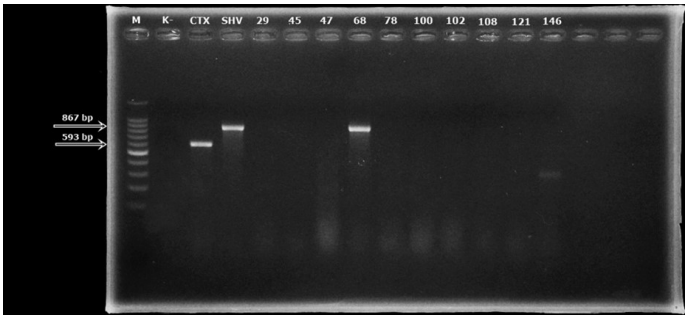

Figure 6. PCR electrophoresis results for the detection of the bla_{SHV} gene encoding the ESBL enzyme in *K. pneumoniae* isolates. M: DNA marker (100 bp ladder); K–: Negative control; CTX: Antibiotic positive control; SHV: Positive control of the *bla*SHV gene.

Detection of the bla_{TEM} gene was performed using the primer pair $_{TEM}$-F (5′-ATGAGTATTCAACATTTCCG-3′) and TEM-R (5′-CTGACAGTTACCAATGCTTA-3′). Meanwhile, detection of the bla_{SHV} gene used the primers SHV-F (5′-GGTTATGCGTTATATTCGCC-3′) and SHV-R (5′-TTAGGTTGCCAGTGCTC-3′). The DNA marker used in this PCR reaction was a DNA ladder from Invitrogen, with a primary reference band at 867 bp.

The amplification results showed positive detection of the bla_{TEM} and bla_{SHV} genes in several *K. pneumoniae* isolates identified as multidrug-resistant (MDR). bla_{TEM} gene amplification produced a DNA band measuring 867 bp. Five MDR isolates (sample codes 29, 68, 78, 102, and 121) showed a clear DNA band at that position, indicating the presence of the bla_{TEM} gene, which plays a role in resistance to β-lactam antibiotics through the production of ESBL enzymes. The other isolates did not show a band at the target size and were therefore considered negative for this gene.

These findings support the phenotypic data on MDR and indicate the potential role of canaries as a reservoir of resistance genes in the environment.

Meanwhile, amplification of the bla_{SHV} gene produced a 593 bp DNA band. Of all the isolates tested, only one MDR isolate (sample code 68) showed positive amplification at this position. This indicates that the bla_{SHV} gene is present in a more limited quantity than bla_{TEM} in the tested samples. These results underscore the importance of molecular monitoring for the presence of various ESBL genes, particularly in the context of the spread of antimicrobial resistance in non-productive animals such as canaries.

Discussion

Based on the isolation results, colonies suspected to be *K. pneumoniae* were obtained with pink characteristics and a mucoid appearance. The mucoid appearance of *K. pneumoniae* colonies is caused by the presence of a polysaccharide capsule, which is an important virulence factor and is closely related to the severity of infection (Pereira and Vanetti, 2015). Of the 150 samples grown on MacConkey Agar (MCA) media, 12 samples were identified as positive as *K. pneumoniae*. The change in the color of the media and colonies to pink on MCA indicates that *K. pneumoniae* is able to ferment lactose into acid, thus causing a change in the color of the pH indicator (neutral red) on the media (Silva-Bea *et al*., 2024). Microscopically, the observed colonies showed characteristics of gram-negative, non-motile, and short rod-shaped bacteria (Lenchenko *et al*., 2020).

Further identification was carried out through a series of IMViC biochemical tests (Indole, Methyl Red, Voges-Proskauer, and Citrate), as well as the Sulfide Indole Motility (SIM) test. This biochemical test aims to determine the identity of bacterial species based on their metabolic and physiological characteristics. In the SIM test, it was found that *K. pneumoniae* is non-motile, indicated by the absence of spread from the inoculation line (Abbas *et al*., 2024). The indole test was conducted to evaluate the ability of bacteria to break down tryptophan into indole (Agustin *et al*., 2024). The results showed that *K. pneumoniae* did not form a red ring on the surface of the media after the addition of Kovac's reagent, indicating an inability to produce indole. In addition, no black color formed on the SIM media, indicating that the *K. pneumoniae* isolate does not produce hydrogen sulfide (H_2S) gas (Thesia *et al*., 2025).

The Methyl Red (MR) test showed a negative result, indicated by no change in the color of the media after the addition of methyl red reagent. This result indicates that *K. pneumoniae* does not produce strong acids as a result of glucose fermentation (Abbas *et al*., 2024). In contrast, the Voges-Proskauer (VP) test gave a positive result, indicated by a change in the color of the media to red after the addition of α-naphthol and KOH reagents. This positive reaction indicates that *K. pneumoniae* is able to ferment glucose and produce neutral compounds such as acetylmethylcarbinol, and has the ability of the buffer system to neutralize acid products (Riwu *et al*., 2022).

The citrate test using Simmons' Citrate Agar (SCA) showed a positive result, indicated by a color change of the bromthymol blue indicator from green to blue. This indicates that *K. pneumoniae* is capable of utilizing citrate as its sole carbon source and inorganic ammonium salts as its sole nitrogen source (Xu *et al*., 2025).

Biochemical tests using Triple Sugar Iron Agar (TSIA) media showed a change in the color of the media to yellow overall and the formation of gas. These results indicate that *K. pneumoniae* has the ability to ferment several types of carbohydrates such as glucose, lactose, maltose, mannitol, and sucrose, which produce acid and gas. The absence of black color in the media indicates that the isolate does not produce hydrogen sulfide (H_2S) gas (Wibisono *et al*., 2020). In addition, the results of antibiotic resistance tests on *K. pneumoniae* isolates showed the presence of a bacterial growth inhibition zone around the antibiotic disk. A total of 10 isolates (83.3%) were identified as MDR, which is resistant to three or more classes of antibiotics.

Research conducted by Permatasari *et al*. (2020) reported that *Klebsiella* sp. isolates obtained from chicken cloacal swabs showed resistance to several antibiotics, including ampicillin, erythromycin, tetracycline, and sulfamethoxazole. Meanwhile, Rueanghiran *et al*. (2019) reported that *K. pneumoniae* was detected in 17.6% of pet birds and 12.9% of individuals who had direct contact with birds. In Brazil, *K. pneumoniae* was isolated from 8.6% to 17.7% of parrots (Vaz *et al*., 2017), while in Thailand, the prevalence of *K. pneumoniae* isolates reached 8% in psittacine birds with respiratory tract infections (Ahmed *et al*., 2021). *K. pneumoniae* is known as a pathogen in birds from the orders Passeriformes and Psittaciformes, which can be found in the feces of healthy birds but can also potentially cause respiratory tract infections (Davies *et al*., 2016). In humans, *K. pneumoniae* is a major cause of various serious infections, such as meningitis, pneumonia, urinary tract infections, intra-abdominal infections, and skin and soft tissue infections (Vading *et al*., 2018; Ahmed *et al*., 2021).

The results of molecular identification by PCR in this study showed that the bla_{TEM} gene was positively detected in 6 of 10 MDR *K. pneumoniae* isolates. The bla_{TEM} gene encodes a β-lactamase enzyme commonly found in ESBL isolates, especially in *K. pneumoniae* and *Escherichia coli* strains (Agustin *et al*., 2025). Other members of the β-lactamase family, such as bla_{SHV} and bla_{CTX-M}, are also frequently found in bacteria from the Enterobacteriaceae family (Ghenea *et al*., 2022). Previous research by Effendi *et al*. (2022) on ESBL-positive *K. pneumoniae* isolates from food-producing animals using multiplex PCR demonstrated successful amplification of the bla_{TEM} and *bla*CTX-M genes. The results of DNA electrophoresis obtained from these isolates showed specific DNA bands of the bla_{TEM} gene amplified using the bla_{TEM}-F and bla_{TEM}-R primers. Of the 10 samples tested, 9 isolates (90%) showed positive results for the bla_{TEM} gene, which strengthens the findings in this study.

The ability of ESBL-producing strains to hydrolyze β-lactam antibiotics is generally caused by mutations in the gene encoding the β-lactamase enzyme, one of which is bla_{TEM}. Mutations that occur in the active site of the enzyme can increase enzymatic activity against various antibiotics (Ahadini *et al*., 2025). Research conducted by Hassan and Shobrak (2015) reported that the bla_{TEM} gene was detected in 7 of 17 multiple-resistant Enterobacteriaceae isolates, which showed resistance to ampicillin, cephalosporin, or cefotaxime, and was previously also found in wild rabbit feces. Another study by Pishtiwan and Khadija (2019) revealed that the bla_{TEM} gene was the most common gene found, with a prevalence of 81% in *E. coli* isolates and 64.7% in ESBL-producing *K. pneumoniae*.

The β-lactamase enzyme produced by ESBL-carrying bacteria is capable of hydrolyzing β-lactam antibiotics, including third- and fourth-generation cephalosporins (Agumah *et al*., 2025). Several frequently found ESBL gene variants include bla_{TEM}, bla_{SHV}, and bla_{CTX-M}, which are generally located on plasmids and contribute to horizontal transmission between species (Husna *et al*., 2023). Increased interactions between humans, livestock, and wildlife may increase the risk of cross-species transmission of ESBL-producing *K. pneumoniae* (Kabali *et al*., 2021). In this study, of 10 MDR *K. pneumoniae* isolates tested by PCR, one isolate was detected positive for carrying the bla_{SHV} gene. Detection of this gene indicates that isolates originating from imported canary feces have the potential to produce ESBL enzymes. The bla_{SHV} gene was first identified as SHV-1, which was initially found on the plasmid and chromosome of penicillin-resistant *K. pneumoniae* (Doosti *et al*., 2015).

The SHV enzyme produced by bacteria is thought to have been acquired through selection along with the emergence of other resistance genes (Moon and Huang, 2023). Although not experiencing explosive spread like the CTX-M variant, the SHV gene has been found in several Enterobacteriaceae species outside of typical clinical hosts such as *K. pneumoniae* and *E. coli*, with increasing levels of allelic variation (Ghenea *et al*., 2022). bla_{SHV} variants, such as bla_{SHV-12}, have even been detected in imported vegetables in Switzerland, found in *Cronobacter sakazakii*, a foodborne opportunistic pathogen with the potential to cause bactere-

mia and meningitis (Zurfluh *et al*., 2015).

These identification results confirmed the spread of ESBL variants, including bla_{SHV-12} and bla_{CTX-M}, in wild birds across various regions. Although the bla_{SHV} genotype is relatively uncommon, its prevalence in *K. pneumoniae* (35.2%) was higher than in *E. coli* (16.2%) (Pishtiwan and Khadija, 2019). Horn *et al*. (2015) reported that the prevalence of enterobacteria from cloacal swabs of canaries reached 10.85% (42/387), while from necropsy results it was 32.76% (19/58). Therefore, international organizations such as the FAO have begun to pay attention to the relationship between wildlife, humans, and livestock in the context of antimicrobial resistance (Wall *et al*., 2016).

The risk of antimicrobial resistance (AMR) is increasing due to the close relationship between pets and humans, which opens up opportunities for reciprocal transmission of both commensal and pathogenic bacteria (Gwenzi *et al*., 2021). The presence of antibiotic-resistant *K. pneumoniae* in canaries indicates a potential zoonotic risk, given that pet birds are often in close contact with their owners (Ahmed *et al*., 2021). In Brazil, *E. coli* and *K. pneumoniae* were the most frequently isolated bacterial species from canaries, suggesting zoonotic potential given the large number of domestic birds kept in domestic environments and direct interaction with humans (Horn *et al*., 2015).

The findings of this study confirm that *K. pneumoniae* isolated from the feces of imported canaries not only exhibits phenotypic characteristics of MDR bacteria, but also carries the genes encoding the ESBL enzymes bla_{TEM} and bla_{SHV}. The presence of these resistance genes indicates the potential role of pet birds as reservoirs of resistant bacteria that can contribute to the spread of antimicrobial resistance across species, including to humans. Given the high level of interaction between pet birds and their owners, these results highlight the importance of monitoring antibiotic use and routine molecular detection in non-productive animals to anticipate the risk of zoonotic transmission and support antibiotic resistance control efforts within the One Health approach.

Conclusion

Imported canaries can be a reservoir for antibiotic-resistant *K. pneumoniae* and contribute to the spread of antimicrobial resistance across species. Therefore, strict monitoring of imported bird traffic and the implementation of a One Health approach to mitigate the risk of antimicrobial resistance are needed.

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Conflict of interest

The authors have no conflict of interest to declare.

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