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RESEARCH ARTICLE

Molecular detection of the *bla*$_{TEM}$ gene in multidrug-resistant *Klebsiella pneumoniae* isolated from chicken eggshells in traditional markets in Surabaya, Indonesia

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ABSTRACT

Background and Aim: Antimicrobial resistance (AMR) in food-associated bacteria is a growing One Health concern because contaminated animal-derived products may spread resistance determinants. Chicken eggshells can become contaminated during production, transportation, storage, and retail handling, creating opportunities for cross-contamination. This study aimed to detect the *bla*$_{TEM}$ gene in multidrug-resistant (MDR) *Klebsiella pneumoniae* isolated from chicken eggshells obtained from traditional markets in Surabaya, Indonesia.

Materials and Methods: A total of 160 eggshell swab samples, comprising 80 broiler and 80 free-range chicken eggs, were collected from 10 traditional markets in Surabaya from December 2024 to January 2025. Samples were cultured on MacConkey agar, and presumptive isolates were characterized by Gram staining and biochemical testing. Antimicrobial susceptibility was evaluated using the Kirby–Bauer disk diffusion method. Isolates resistant to three or more antimicrobial classes were classified as MDR and subjected to polymerase chain reaction (PCR) targeting an 867-bp fragment of the *bla*$_{TEM}$ gene.

Results: *K. pneumoniae* was detected in 41/160 (25.62%) eggshell samples. Prevalence was significantly higher in broiler eggshells (35.00%; 28/80) than in free-range chicken eggshells (16.25%; 13/80; $\chi^2 = 7.12$, $p = 0.008$). All 41 isolates were resistant to amoxicillin, followed by erythromycin (90.24%), streptomycin (26.82%), ciprofloxacin (14.63%), and tetracycline (7.31%). Fourteen isolates (34.15%) were classified as MDR. PCR detected *bla*$_{TEM}$ in 4/14 (28.57%) MDR isolates, representing 9.76% (4/41) of all *K. pneumoniae* isolates and 2.50% (4/160) of all eggshell samples. All four *bla*$_{TEM}$-positive isolates originated from broiler eggshells.

Conclusion: MDR *K. pneumoniae* carrying *bla*$_{TEM}$ was detected on chicken eggshells from traditional markets, demonstrating

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the occurrence of an important β-lactam resistance determinant at the poultry food–consumer interface. Although the overall frequency of *bla*$_{TEM}$-positive samples was low, contaminated eggshells may provide opportunities for cross-contamination during handling. Broader surveillance incorporating phenotypic extended-spectrum β-lactamase confirmation, additional resistance genes, and molecular characterization is warranted to clarify transmission dynamics and public health significance.

Keywords: antimicrobial resistance, *bla*$_{TEM}$, chicken eggshell, extended-spectrum β-lactamase, *Klebsiella pneumoniae*, multidrug resistance, One Health, traditional market.

INTRODUCTION

Antimicrobial resistance (AMR) has emerged as a critical global public health threat, undermining effective treatment of infectious diseases in humans and animals [1]. The emergence and spread of MDR Enterobacteriaceae, particularly Gram-negative pathogens resistant to critically important antimicrobial agents, have raised substantial global concerns [2]. The World Health Organization (WHO) has identified carbapenem- and third-generation cephalosporin-resistant Enterobacteriaceae as critical-priority pathogens, emphasizing the urgent need for effective therapeutic and surveillance strategies [3].

Among these pathogens, *Klebsiella pneumoniae* is a key member of the ESKAPE group, which includes bacterial pathogens that evade antimicrobial treatment and cause severe healthcare-associated infections [4, 5]. *K. pneumoniae* is a nonmotile, rod-shaped, Gram-negative bacterium surrounded by a protective polysaccharide capsule and is recognized as an opportunistic pathogen [6]. It commonly colonizes the intestinal and respiratory tracts of humans and animals as part of the normal microbiota [7]. However, under favorable conditions, *K. pneumoniae* can cause a wide range of infections, including urinary tract infections, pneumonia, sepsis, and skin and mucosal infections [8].

A major concern associated with *K. pneumoniae* is its remarkable capacity to acquire and disseminate AMR through multiple mechanisms [9]. In Gram-negative bacteria, AMR can arise through mechanisms such as enzymatic degradation of antimicrobial agents, modification of target sites, and alterations in metabolic pathways [10]. Two particularly important resistance mechanisms in *K. pneumoniae* are carbapenemase production and extended-spectrum β-lactamase (ESBL) synthesis, which substantially reduce susceptibility to β-lactam antimicrobial agents, including penicillins, cephalosporins, and monobactams [11]. Furthermore, *K. pneumoniae* frequently harbors plasmid-mediated ESBL-associated genes, including *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$, which can contribute to the horizontal dissemination of AMR determinants [12].

ESBL-producing *K. pneumoniae* in the food chain, including poultry products, retail meat, milk, and eggs, has raised concerns about potential foodborne transmission pathways [13]. From a One Health perspective, the dissemination of antimicrobial-resistant bacteria through the food chain is particularly relevant to poultry production systems, in which antimicrobial use and the subsequent emergence and circulation of resistant bacteria remain important concerns [14]. Poultry eggs, particularly their shell surfaces, can become contaminated during laying, transportation, storage, and retail handling and may therefore serve as an interface through which bacteria are transferred between poultry production environments, food handling environments, and consumers [15].

Despite increasing attention to antimicrobial-resistant bacteria in poultry and poultry-derived foods, information on ESBL-associated *K. pneumoniae* contamination of chicken eggshells in Indonesia remains limited. Previous research in Surabaya traditional markets has investigated MDR *K. pneumoniae* from chicken eggshell swabs, including molecular detection of the *bla*$_{SHV}$ gene [16]. However, evidence specifically addressing the occurrence of *bla*$_{TEM}$-positive MDR *K. pneumoniae* on chicken eggshell surfaces remains limited. This knowledge gap is particularly relevant in Surabaya, where traditional markets represent important points in the poultry food distribution chain and where variations in sanitation, storage, and repeated handling may facilitate bacterial contamination and cross-contamination [17]. Therefore, determining whether MDR *K. pneumoniae* carrying *bla*$_{TEM}$ is present on eggshell surfaces is important for generating baseline molecular epidemiological data and improving understanding of the potential role of poultry-derived food-contact surfaces in the circulation of AMR determinants.

The *bla*$_{TEM}$ gene was selected as the molecular target because TEM β-lactamases are widely reported among Gram-negative bacteria, and *bla*$_{TEM}$ represents an important resistance determinant for molecular surveillance of β-lactam resistance [18]. Although other clinically relevant ESBL-associated genes, including *bla*$_{SHV}$ and *bla*$_{CTX-M}$, may also occur in *K. pneumoniae*, targeted investigation of *bla*$_{TEM}$ provides complementary molecular information for surveillance of resistance determinants in poultry-associated samples [19]. Because the present study targeted

bla$_{TEM}$ and did not include phenotypic confirmation of ESBL production, detection of this gene was interpreted as evidence of an ESBL-associated resistance determinant rather than definitive confirmation of an ESBL-producing phenotype. Its detection among MDR isolates from eggshell surfaces may therefore provide locally relevant evidence regarding the occurrence of β-lactam resistance determinants at the poultry food–consumer interface.

This study hypothesized that chicken eggshells sold in traditional markets in Surabaya may harbor MDR *K. pneumoniae* carrying the *bla*$_{TEM}$ gene. Accordingly, the study aimed to isolate and identify *K. pneumoniae* from broiler and free-range chicken eggshell swabs collected from traditional markets in Surabaya, Indonesia; determine the antimicrobial susceptibility profiles and identify MDR isolates; and molecularly detect the *bla*$_{TEM}$ gene among the identified MDR *K. pneumoniae* isolates. The findings were intended to provide baseline evidence of *bla*$_{TEM}$-positive MDR *K. pneumoniae* on chicken eggshell surfaces, rather than to establish phenotypically confirmed ESBL-producing isolates, and to contribute to AMR surveillance within a One Health framework.

MATERIALS AND METHODS

Ethical approval

This study involved food-contact environmental samples collected as swabs from chicken eggshell surfaces at retail markets and did not involve live animals or human participants. Therefore, formal ethical approval was not required in accordance with institutional guidelines. Sample collection involved only commercially available eggs and did not include any intervention involving animals. All procedures for sample handling, bacterial isolation, antimicrobial susceptibility testing, and molecular analysis were conducted using Biosafety Level 2 (BSL-2) laboratory practices and in accordance with standard microbiological safety protocols at the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia.

Study period and location

The study was conducted from December 2024 to January 2025 in Surabaya, Indonesia. Eggshell swab samples were collected from 10 traditional markets: Pacar Keling, Manyar, Pabean, Kapas Krampung, Sememi, Pucang, Keputran, Simo, Wiyung, and Wonokromo. The markets were selected using convenience sampling based on accessibility, market size, and their representation as major traditional poultry retail points across Surabaya.

Bacterial isolation, identification, and antimicrobial susceptibility testing were performed at the Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga. Molecular detection of the *bla*$_{TEM}$ gene by polymerase chain reaction (PCR) was performed at the Institute of Tropical Disease Laboratory, Universitas Airlangga.

Study design

This cross-sectional study investigated the occurrence of presumptive *K. pneumoniae* carrying the *bla*$_{TEM}$ gene on chicken eggshell surfaces in traditional markets in Surabaya. A total of 160 eggshell swab samples, comprising 80 broiler chicken eggs and 80 free-range chicken eggs, were collected from 10 traditional markets. Samples were subjected to bacterial isolation and conventional biochemical identification followed by antimicrobial susceptibility testing. Isolates meeting the predefined MDR criteria were subsequently examined for the presumptive molecular detection of the *bla*$_{TEM}$ gene. The prevalence of *K. pneumoniae*, MDR isolates, and *bla*$_{TEM}$-positive isolates was compared between broiler and free-range chicken egg samples.

Sample collection

A total of 160 eggshell swab samples were analyzed, comprising 80 broiler chicken eggs and 80 free-range chicken eggs. Only visibly intact, noncracked eggs were included. Eggshell surface conditions (clean or dirty) were recorded at the time of sampling, whereas visibly washed eggs were excluded to minimize potential bias associated with cleaning before sampling.

The criteria used to classify eggs as broiler or free-range chicken eggs should reflect the actual information available at the point of collection. The number of vendors sampled per market, the number of eggs obtained from each vendor, and the procedure used to select vendors and individual eggs were not specified in the available study records.

The outer surface of each eggshell was gently swabbed using a sterile cotton swab premoistened with physiological saline. The sampled surface area and the number and direction of swabbing strokes were not documented in the available methodological information. Each swab was subsequently placed in a test tube containing 1% buffered peptone water (BPW; Oxoid, UK), which served as the transport medium. All samples were

transported in a cooled insulated container at 4–8°C and processed within 4 h of collection. Pre-enrichment was performed by incubating the BPW tubes at 37°C for 18–24 h before plating [17].

Isolation and identification of *K. pneumoniae*

Following pre-enrichment, BPW cultures were streaked onto MacConkey agar (MCA; HiMedia, India, MH081) and incubated at 37°C for 24 h. Presumptive *K. pneumoniae* colonies were identified based on their characteristic large, round, pink, and mucoid appearance on MCA. Presumptive isolates were further characterized by Gram staining and biochemical testing using sulfide-indole-motility (SIM) medium, triple sugar iron agar (TSIA), methyl red–Voges–Proskauer (MR-VP) tests, Simmons citrate agar, and urease tests [16].

Presumptive identification was based on the combined results of colony morphology, Gram staining, and conventional biochemical reactions. Presumptive *K. pneumoniae* isolates exhibited Gram-negative bacilli, lactose-fermenting mucoid colonies, nonmotility, a negative indole reaction, and positive citrate, urease, and Voges–Proskauer reactions.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and *16S ribosomal RNA* (rRNA) gene sequencing were not performed. Therefore, species identity was not independently confirmed using advanced identification or molecular sequencing methods, and the isolates are described as presumptive *K. pneumoniae*.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was evaluated using the Kirby–Bauer disk diffusion method. The antimicrobial disks (Oxoid) tested were ciprofloxacin (5 μg), streptomycin (10 μg), tetracycline (30 μg), erythromycin (15 μg), and amoxicillin (25 μg). These agents represent the fluoroquinolone, aminoglycoside, tetracycline, macrolide, and penicillin subclasses of the β-lactam class, respectively. Inhibition zones were interpreted according to the applicable Clinical and Laboratory Standards Institute (CLSI) guidelines [20], where valid interpretive criteria were available for the organism–antimicrobial combination.

Standardized clinical breakpoints for erythromycin against Enterobacterales are not generally established for routine clinical susceptibility interpretation. Therefore, erythromycin results should not be classified as susceptible or resistant without an appropriate, validated interpretive criterion and supporting reference. Similarly, the applicability of interpretive criteria to streptomycin and amoxicillin must be verified before assigning categorical susceptibility results. Where validated breakpoints are unavailable, inhibition-zone measurements should be reported descriptively rather than assigned unsupported susceptibility categories.

All antimicrobial susceptibility tests were performed in duplicate to ensure reproducibility. *Escherichia coli* American Type Culture Collection 25922 was used as the quality-control reference strain for disk diffusion testing.

MDR was defined as acquired non-susceptibility to at least one antimicrobial agent in three or more antimicrobial classes, provided that the definition was supported by reference [21] and that valid interpretive criteria were available for the agents included in the classification. The MDR classification was based on distinct antimicrobial classes rather than the number of individual antimicrobial agents. The treatment of intermediate susceptibility results should follow the criteria specified in the applicable reference.

No phenotypic ESBL screening or confirmatory testing was performed. Consequently, the ESBL phenotype of the recovered isolates was not determined, and detection of the blaTEM gene was not interpreted as confirmation of ESBL production. Only isolates meeting the applicable MDR criterion were subsequently subjected to PCR.

Molecular detection of the *bla*$_{TEM}$ gene using PCR

PCR analysis for the *bla*$_{TEM}$ gene was performed on MDR *K. pneumoniae* isolates. DNA was extracted using the boiling method. DNA concentration and purity were measured using a NanoDrop spectrophotometer, and 50-100 ng of template DNA was used per reaction.

PCR was performed in duplicate for each isolate. A no-template control, extraction blank, and positive amplification control were included in each run to monitor contamination and PCR inhibition. The primer sequences used for detection of the *bla*$_{TEM}$ gene, together with the annealing temperature and expected amplicon size, are presented in Table 1 [22].

The total PCR reaction volume, reagent composition, primer concentrations, individual reagent volumes, and complete thermocycling conditions were not specified in the available methodological information. These parameters must be reported from the original laboratory protocol to ensure reproducibility.

A previously confirmed *bla*$_{TEM}$-positive clinical *K. pneumoniae* isolate from the laboratory collection was used

as the positive control for *bla*$_{TEM}$ amplification. The source of this isolate and the method by which its *bla*$_{TEM}$-positive status was previously established were not specified in the available records. PCR products were visualized using 2% agarose gel electrophoresis. Amplicons producing a band corresponding to the expected size of 867 bp were considered presumptively positive for *bla*$_{TEM}$.. Sequencing confirmation of the amplified product or identification of the specific *bla*$_{TEM}$ variant was not performed.

Table 1: Primers used for *bla*$_{TEM}$ gene detection.

Target gene	Primer sequence	Annealing temperature	Amplicon (bp)	Reference
bla$_{TEM}$	Forward: 5′-ATGAGTATTCAACATTTCCG-3′ Reverse: 5′-CTGACAGTTACCAATGCTTA-3′	58°C	867	[22]

Statistical analysis

Statistical analyses were performed using SPSS version 26.0 (IBM Corp., USA). The prevalence of *K. pneumoniae*, MDR isolates, and *bla*$_{TEM}$-positive isolates was summarized as frequencies and percentages. The denominator for each proportion should match the outcome being analyzed: eggshell samples for sample-level occurrence, recovered presumptive K. pneumoniae isolates for the MDR proportion, and tested MDR isolates for the presumptive *bla*$_{TEM}$-positive proportion.

Comparisons between broiler and free-range chicken egg samples were performed using the chi-square test when the expected cell frequencies met the applicable assumptions. Fisher's exact test was used when expected cell frequencies were too small for reliable chi-square approximation. The specific test used for each comparison should correspond to the actual contingency tables and statistical output.

Prevalence estimates were accompanied by 95% confidence intervals (CIs). The method used to calculate the CIs must be specified according to the actual statistical procedure. Statistical significance was set at $p < 0.05$.

RESULTS

Isolation and identification

Of the 160 chicken eggshell swab samples examined, 41 (25.62%) were positive for *K. pneumoniae*, comprising 28 isolates from broiler chicken eggshells and 13 isolates from free-range chicken eggshells (Table 2). *K. pneumoniae* prevalence was significantly higher in broiler chicken eggshell samples (35.00%; 28/80) than in free-range chicken eggshell samples (16.25%; 13/80). The Chi-square test showed a significant difference between the two egg types ($\chi^2 = 7.12$, $p = 0.008$).

Table 2: Prevalence and distribution of *Klebsiella pneumoniae* isolated from chicken eggshell swab samples collected from traditional markets in Surabaya.

Egg type	Market of origin	Number of samples	Positive (n)	Percentage
Broiler chicken eggshell	Keputran	8	2	25.00
	Pacar Keling	8	3	37.50
	Manyar	8	3	37.50
	Pucang	8	3	37.50
	Kapas Krampung	8	1	12.50
	Pabean	8	3	37.50
	Wonokromo	8	5	62.50
	Wiyung	8	2	25.00
	Simo	8	3	37.50
	Sememi	8	3	37.50
	Subtotal	80	28	35.00
Free-range chicken eggshell	Keputran	8	1	12.50
	Pacar Keling	8	2	25.00
	Manyar	8	1	12.50
	Pucang	8	1	12.50
	Kapas Krampung	8	1	12.50
	Pabean	8	0	0.00
	Wonokromo	8	2	25.00
	Wiyung	8	1	12.50
	Simo	8	1	12.50
	Sememi	8	3	37.50
	Subtotal	80	13	16.25
Total		160	41	25.62

K. pneumoniae-positive samples were distributed across the traditional markets as follows: Keputran, 3; Pacar Keling, 5; Manyar, 4; Pucang, 4; Kapas Krampung, 2; Pabean, 3; Wonokromo, 7; Wiyung, 3; Simo, 4; and Sememi, 6.

On MCA, presumptive *K. pneumoniae* colonies appeared large, round, pink, smooth, glistening, and distinctly mucoid, consistent with lactose fermentation (Figure 1). Biochemical characterization showed an acid/acid reaction with gas production on TSIA, nonmotility on SIM medium, negative indole and methyl red reactions, and positive Voges–Proskauer, citrate, and urease reactions. Because identification relied on culture characteristics and conventional biochemical tests, the isolates were considered presumptive *K. pneumoniae* rather than molecularly confirmed species.



Figure 1: Representative image of smooth, round, pink, mucoid lactose-fermenting colonies presumptively identified as *Klebsiella pneumoniae* on MacConkey agar after 24 h of incubation at 37°C.

AMR profiles

Antimicrobial susceptibility testing showed that all *K. pneumoniae* isolates were resistant to amoxicillin (100.00%; 41/41). Resistance was also observed to erythromycin (90.24%; 37/41), streptomycin (26.82%; 11/41), ciprofloxacin (14.63%; 6/41), and tetracycline (7.31%; 3/41). No intermediate susceptibility results were observed for the tested antimicrobial agents; all isolates were categorized as either resistant or susceptible according to the interpretive criteria applied in this study. The erythromycin classifications require cautious interpretation because an applicable standardized clinical breakpoint for Enterobacterales was not established in the information provided.

Resistance profiles according to egg type are presented in Table 3 and Figure 2. Among isolates from broiler chicken eggshells, resistance to amoxicillin was observed in 100.00% (28/28), erythromycin in 89.28% (25/28), streptomycin in 32.14% (9/28), ciprofloxacin in 17.85% (5/28), and tetracycline in 7.14% (2/28). Among isolates obtained from free-range chicken eggshells, resistance was observed to amoxicillin in 100.00% (13/13), erythromycin in 92.30% (12/13), streptomycin in 15.38% (2/13), ciprofloxacin in 7.69% (1/13), and tetracycline in 7.69% (1/13). The reported percentages indicate that resistance was not consistently higher among broiler-derived isolates across all tested agents. Differences in streptomycin and ciprofloxacin resistance frequencies between isolates from broiler and free-range chicken eggshells were not statistically significant ($p > 0.05$). Exact p-values and the statistical tests used for each comparison should be provided if these analyses were performed.

Table 3: Antimicrobial susceptibility profile of *Klebsiella pneumoniae* isolates from broiler and free-range chicken eggshell samples based on Clinical and Laboratory Standards Institute interpretation criteria

Antibiotic	Broiler resistant, n (%)	Free-range resistant, n (%)	Total resistant, n (%)	Intermediate, n (%)	Susceptible, n (%)
Amoxicillin	28 (100.00)	13 (100.00)	41 (100.00)	0 (0.00)	0 (0.00)
Erythromycin	25 (89.29)	12 (92.31)	37 (90.24)	0 (0.00)	4 (9.76)
Streptomycin	9 (32.14)	2 (15.38)	11 (26.83)	0 (0.00)	30 (73.17)
Ciprofloxacin	5 (17.86)	1 (7.69)	6 (14.63)	0 (0.00)	35 (85.37)
Tetracycline	2 (7.14)	1 (7.69)	3 (7.32)	0 (0.00)	38 (92.68)

Among the 41 *K. pneumoniae* isolates, 14 (34.15%; 14/41) met the study criterion for MDR. The proportion of MDR isolates was 39.29% (11/28) among isolates from broiler chicken eggshells and 23.08% (3/13) among those from free-range chicken eggshells. These proportions are presented descriptively because a statistical comparison

between egg categories was not documented in the supplied results. The complete resistance profiles of the 14 MDR isolates are presented in Table 4.

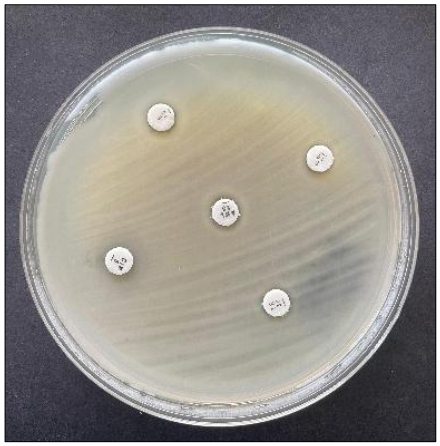


Figure 2: Representative Kirby–Bauer disk diffusion assay showing a multidrug-resistant *Klebsiella pneumoniae* isolate with reduced inhibition zones. Disk positions: (1) amoxicillin, (2) tetracycline, (3) streptomycin, (4) erythromycin, and (5) ciprofloxacin.

Table 4: Complete multidrug resistance patterns of the 14 *Klebsiella pneumoniae* isolates recovered from eggshell swab samples

No.	Sample code	Egg type	Resistance pattern
1	NCPK7	Broiler	AML/S/E
2	KCK5	Free-range	AML/S/E
3	NWSE8	Broiler	AML/S/E
4	NWSI1	Broiler	AML/S/E
5	NEM2	Broiler	AML/S/E
6	KEM2	Free-range	AML/S/E
7	NEP3	Broiler	AML/S/E
8	NWSE2	Broiler	AML/E/CIP
9	NWSI5	Broiler	AML/E/CIP
10	NWSI7	Broiler	AML/S/E/CIP
11	NEM7	Broiler	AML/S/E/CIP
12	NEM4	Broiler	AML/TE/S/E
13	KSW2	Free-range	AML/TE/E/CIP
14	NCK5	Broiler	AML/TE/S/E/CIP

AML = amoxicillin; CIP = ciprofloxacin; E = erythromycin; S = streptomycin; TE = tetracycline.

Molecular detection of the *bla*$_{TEM}$ gene

PCR analysis of the 14 MDR *K. pneumoniae* isolates showed that four isolates (28.57%; 4/14) produced an amplification product corresponding to the expected 867-bp size for *bla*$_{TEM}$ (Figure 3 and Table 5). These isolates were classified as presumptively blaTEM-positive because the amplification products were not sequenced. The four presumptively *bla*$_{TEM}$-positive isolates were NEM4 from a broiler chicken egg sampled at Manyar, NWSI1 and NWSI7 from broiler chicken eggs sampled at Simo, and NWSE2 from a broiler chicken egg sampled at Sememi. No *bla*$_{TEM}$-positive isolate was detected among isolates obtained from free-range chicken eggshells.

Relative to all *K. pneumoniae* isolates, the prevalence of presumptively *bla*$_{TEM}$-positive isolates was 9.76% (4/41). Relative to all eggshell swab samples examined, the prevalence was 2.50% (4/160). These findings indicate the detection of amplification products of the expected size in four MDR isolates; they do not establish sequence-confirmed detection or identify a specific *bla*$_{TEM}$ variant.

DISCUSSION

Occurrence of *K. pneumoniae* on chicken eggshells

In the present study, presumptive *K. pneumoniae* was detected in 41 of 160 chicken eggshell swab samples (25.62%), comprising 28 isolates from broiler chicken eggshells and 13 isolates from free-range chicken eggshells. The prevalence was significantly higher in broiler chicken eggshell samples than in free-range chicken eggshell samples (35.00% vs. 16.25%; $\chi^2 = 7.12$, $p = 0.008$). This difference may reflect variation in production systems, stocking density, environmental hygiene, and egg-handling practices [24]; however, the present study did not

directly assess these factors. Intensive poultry production environments may involve greater animal density and opportunities for bacterial persistence and cross-contamination of egg surfaces [25]. In contrast, free-range production systems may involve different environmental exposure and handling conditions that influence eggshell contamination [26].

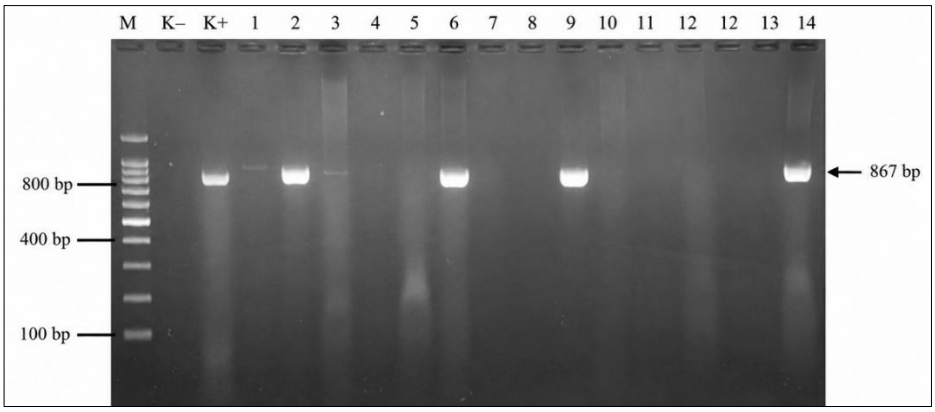


Figure 3: Agarose gel electrophoresis of PCR products showing amplification products at the expected 867-bp size for the *bla*$_{TEM}$ gene. Lane M: 100-bp DNA ladder; lane K−: negative control; lane K+: positive control; lanes 1–14: MDR isolates. Bands at the expected size were observed in lanes 2 (NEM4), 6 (NWSI1), 9 (NWSI7), and 14 (NWSE2). The amplicons were not sequenced; therefore, sequence analysis did not confirm their identity.

Table 5: Polymerase chain reaction detection of the *bla*$_{TEM}$ gene among multidrug-resistant *Klebsiella pneumoniae* isolates according to egg type and market origin

No.	Sample code	Egg type	Market of origin	*bla*$_{TEM}$
1	NEM2	Broiler chicken	Manyar	−
2	NEM4	Broiler chicken	Manyar	+
3	NEM7	Broiler chicken	Manyar	−
4	KSW2	Free-range chicken	Wonokromo	−
5	KEM2	Free-range chicken	Manyar	−
6	NWSI1	Broiler chicken	Simo	+
7	NWSI5	Broiler chicken	Simo	−
8	NWSE8	Broiler chicken	Sememi	−
9	NWSI7	Broiler chicken	Simo	+
10	NCK5	Broiler chicken	Keputran	−
11	KCK5	Free-range chicken	Keputran	−
12	NEP3	Broiler chicken	Pucang	−
13	NCPK7	Broiler chicken	Pacar Keling	−
14	NWSE2	Broiler chicken	Sememi	+
Total positive				4

+ = positive; − = negative.

The overall prevalence observed in this study was comparable to the 25.80% prevalence of *K. pneumoniae* previously reported in broiler chicken cecal samples [27]. However, this comparison should be interpreted cautiously because cecal specimens and eggshell swabs represent different biological sample types. Similarly, *Klebsiella* spp. contamination of eggshells has been reported at 24% in traditional markets compared with 4% in modern markets [28]. In contrast, a lower prevalence of *K. pneumoniae* (7.80%) was reported on poultry farms in China [29]. Differences among studies may reflect variations in sample type, production system, geographic location, hygiene conditions, sampling strategy, and bacterial detection methods.

In Indonesia, previous studies from East Java and Surabaya have investigated bacterial contamination and AMR in poultry-associated samples; however, molecular evidence specifically addressing presumptively *bla*$_{TEM}$-positive isolates among presumptive MDR *K. pneumoniae* from chicken eggshell surfaces remains limited [15, 16]. The present findings therefore provide additional local evidence on the occurrence of presumptive *K. pneumoniae* and a PCR amplicon of the expected size for *bla*$_{TEM}$ on eggshell surfaces in traditional market settings. *K. pneumoniae* is an opportunistic bacterium found in diverse environmental and host-associated sources, including soil, animal skin, intestinal contents, and feces, and may contaminate foods and other animal-derived products [30].

AMR profiles

Antimicrobial susceptibility testing showed that all presumptive *K. pneumoniae* isolates were resistant to

amoxicillin (100.00%; 41/41), followed by erythromycin (90.24%; 37/41), streptomycin (26.83%; 11/41), ciprofloxacin (14.63%; 6/41), and tetracycline (7.32%; 3/41). The universal resistance to amoxicillin indicates a high frequency of reduced susceptibility to this β-lactam antimicrobial among the isolates examined [31]. However, these findings alone do not establish the source or magnitude of antimicrobial selection pressure within the poultry production or marketing chain.

The high frequency of erythromycin resistance requires particularly cautious interpretation. Macrolides generally have limited activity against Gram-negative Enterobacteriaceae because of intrinsic factors, including low outer-membrane permeability and efflux activity [32]. Therefore, the high frequency of erythromycin nonsusceptibility observed in this study may reflect intrinsic or baseline reduced susceptibility rather than exclusively acquired AMR mechanisms [9]. Consequently, erythromycin resistance should be interpreted cautiously, particularly because the applicability of the interpretive breakpoint used in this study requires clarification. Its contribution to the MDR classification should be reconsidered if the criterion used cannot be adequately justified.

Resistance frequencies were numerically higher for streptomycin and ciprofloxacin in isolates from broiler chicken eggshells, whereas erythromycin and tetracycline resistance frequencies were slightly higher in isolates from free-range chicken eggshells. Differences in streptomycin and ciprofloxacin resistance between the two groups were not statistically significant ($p > 0.05$). Therefore, the findings do not support a general conclusion that isolates from broiler chicken eggshells exhibited consistently higher resistance across all antimicrobial agents. Previous research reported 27.3% ciprofloxacin resistance and 100% resistance to ampicillin and amoxicillin among *K. pneumoniae* isolates [33], demonstrating that substantial β-lactam resistance has also been documented in poultry-associated isolates.

Among the 41 presumptive *K. pneumoniae* isolates, 14 (34.15%) met the study criterion for MDR. The proportion of MDR isolates was numerically higher among isolates from broiler chicken eggshells (39.29%; 11/28) than among those from free-range chicken eggshells (23.08%; 3/13). However, in the absence of a reported statistical comparison between these proportions, this difference should be interpreted descriptively and should not be considered evidence that the production system directly influenced MDR occurrence. The presence of MDR isolates presumptively identified as *K. pneumoniae* is nevertheless relevant because multidrug resistance can restrict antimicrobial treatment options for infections caused by resistant strains [35]. ESBLs and carbapenemases represent important mechanisms through which *K. pneumoniae* can acquire resistance to β-lactam antimicrobial agents [11]. However, this study did not identify a phenotypic ESBL-producing isolate.

Molecular detection of *bla*$_{TEM}$

Among the 14 MDR isolates presumptively identified as *K. Pneumoniae* subjected to PCR, four (28.57%) produced an expected 867-bp blaTEM amplicon. This detection frequency was lower than that reported in previous research [36]. Relative to all presumptive *K. pneumoniae* isolates, presumptively *bla*$_{TEM}$-positive isolates represented 9.76% (4/41), whereas the prevalence among all eggshell swab samples examined was 2.50% (4/160). Therefore, these findings demonstrate the occurrence of MDR isolates presumptively identified as *K. pneumoniae* that were presumptively *bla*$_{TEM}$-positive on chicken eggshell surfaces but do not establish chicken eggs as major reservoirs of antimicrobial-resistant bacteria [28].

Detecting an amplicon of the expected size for *bla*$_{TEM}$ provides evidence consistent with a *bla*$_{TEM}$-related target among the MDR isolates examined [37]. However, because the PCR products were not sequenced and phenotypic ESBL confirmation was not performed, the findings should not be interpreted as confirmation of a specific *bla*$_{TEM}$ variant or of phenotypic ESBL production. Furthermore, the study screened only MDR isolates; therefore, the occurrence of *bla*$_{TEM}$ among non-MDR isolates presumptively identified as *K. pneumoniae* remains unknown.

Public health and One Health implications

From a food safety perspective, the main concern with contaminated eggshells is cross-contamination during handling rather than consumption of adequately cooked eggs, because proper cooking reduces the risk posed by viable vegetative bacterial cells [38]. Contaminated eggshell surfaces may transfer bacteria to hands, kitchen utensils, food preparation surfaces, and ready-to-eat foods during handling [39]. Plasmid-associated β-lactamase genes, including *bla*$_{SHV}$ and *bla*$_{TEM}$, are relevant to AMR because mobile genetic elements can facilitate their dissemination among Gram-negative bacteria [40].

From a One Health perspective, the detection of MDR isolates presumptively identified as *K. pneumoniae*,

including presumptively *bla*$_{TEM}$-positive isolates, on eggshell surfaces highlights a potential interface among poultry production, the market environment, food handling, and human exposure [41]. Eggshell contamination may occur at different stages of the production and distribution chain through contact with fecal material, litter, contaminated equipment, transport environments, storage surfaces, or handlers [42]. However, this cross-sectional study did not trace isolates across these stages and therefore cannot establish a farm-to-table transmission pathway. The findings indicate a potential food-chain interface but do not demonstrate transmission from poultry farms to markets or consumers.

Eggshell surfaces may nevertheless represent an interface connecting animal health, environmental contamination, food safety, and human health [43]. AMR determinants can disseminate among bacterial populations through horizontal gene transfer mechanisms, including conjugation, transformation, and transduction [44]. Traditional market conditions involving repeated handling, mixed storage, open display, and variable sanitation may provide opportunities for bacterial contamination and cross-contamination [45]. ESBL-producing bacteria and associated resistance determinants have also been reported in environmental matrices and food-related sources, demonstrating the broader environmental dimension of AMR [46]. Nevertheless, because the present study did not perform phenotypic ESBL confirmation, the findings should not be interpreted as direct evidence of ESBL-producing bacteria on the sampled eggshells.

These findings support the value of integrated surveillance across poultry production, food-market environments, and public health sectors within a One Health framework [47]. Practical measures may include improved hygiene and sanitation in traditional markets, regular cleaning and disinfection of egg-contact and storage surfaces, appropriate hand hygiene among handlers, rational antimicrobial use in poultry production, and consumer education regarding handwashing and prevention of cross-contamination during food preparation [48].

Study limitations

Several limitations should be considered when interpreting these findings. First, antimicrobial susceptibility testing included only five antimicrobial agents and therefore did not provide a comprehensive resistance profile of the isolates. In addition, the interpretive criterion used for erythromycin requires clarification, as it may affect resistance classification and the MDR assessment. Second, molecular screening was limited to *bla*$_{TEM}$; it did not investigate other clinically relevant ESBL-associated genes, including *bla*$_{SHV}$ and *bla*$_{CTX-M}$. Third, phenotypic ESBL confirmation using a double-disk synergy or combined-disk test was not performed, preventing direct confirmation of ESBL expression at the phenotypic level.

Fourth, the 867-bp PCR products were not sequenced; therefore, the identity and specific variant of *bla*$_{TEM}$ could not be sequence-confirmed. Accordingly, PCR findings were interpreted as presumptive detection based on the expected amplicon size rather than sequence-confirmed identification of *bla*$_{TEM}$ or any specific variant. Fifth, advanced molecular typing methods, such as multilocus sequence typing or whole-genome sequencing, were not performed, preventing assessment of clonal relationships, strain diversity, and the broader genetic context of resistance determinants. Sixth, species-level identification relied on conventional culture and biochemical characteristics because advanced confirmatory methods were unavailable. Thus, the bacterial identification should be interpreted as presumptive because neither MALDI-TOF MS nor 16S rRNA gene sequencing was performed. Finally, sampling was restricted to a single period from December 2024 to January 2025 and to selected traditional markets in Surabaya; therefore, temporal or seasonal variation could not be assessed, and the findings should not be generalized beyond the sampled population without further investigation. Furthermore, because PCR screening was restricted to MDR isolates, the prevalence of *bla*$_{TEM}$ among non-MDR isolates remains undetermined.

CONCLUSION

Presumptive *K. pneumoniae* was detected in 25.62% (41/160) of chicken eggshell swab samples collected from traditional markets in Surabaya, with a significantly higher prevalence in broiler chicken eggshells (35.00%; 28/80) than in free-range chicken eggshells (16.25%; 13/80; $\chi^2 = 7.12$, $p = 0.008$). Among the 41 presumptively identified isolates, 14 (34.15%) met the study criterion for MDR. Four of the 14 MDR isolates (28.57%) produced the expected 867-bp PCR amplicon for *bla*$_{TEM}$, corresponding to 9.76% (4/41) of all presumptive *K. pneumoniae* isolates and 2.50% (4/160) of all eggshell samples examined. All four presumptively *bla*$_{TEM}$-positive isolates originated from broiler chicken eggshells.

The detection of MDR isolates presumptively identified as *K. pneumoniae* and presumptively *bla*$_{TEM}$-positive on eggshell surfaces highlights the importance of hygiene throughout egg handling, transportation, storage, retail

display, and food preparation. Because eggshells may facilitate bacterial transfer to hands, utensils, food-contact surfaces, and other foods, preventive measures should emphasize appropriate market sanitation, cleaning of egg-contact surfaces, handler hand hygiene, rational antimicrobial use in poultry production, and consumer practices that minimize cross-contamination.

This study provides local data on the occurrence of MDR isolates presumptively identified as *K. pneumoniae* and presumptive molecular detection of the bla_{TEM} resistance determinant on chicken eggshell surfaces from multiple traditional markets in Surabaya. The inclusion of both broiler and free-range chicken eggs enabled comparison between the two sampled egg types. The combination of conventional bacterial identification, antimicrobial susceptibility testing, and targeted molecular detection also provided complementary phenotypic and molecular information on the isolates examined.

Future studies should include broader antimicrobial panels, phenotypic ESBL confirmation, and simultaneous molecular screening for multiple resistance determinants, particularly bla_{TEM}, bla_{SHV}, and bla_{CTX-M}. Sequencing of PCR products and advanced genomic approaches should be incorporated to confirm resistance genes, characterize their variants and genetic contexts, and investigate possible clonal relationships among isolates. Longitudinal and multisite surveillance encompassing poultry farms, transportation systems, retail markets, eggshells, environmental samples, and relevant food-contact surfaces would further clarify potential contamination pathways and the epidemiology of AMR within the poultry food chain.

The findings demonstrate the occurrence of MDR isolates presumptively identified as *K. pneumoniae* and presumptively bla_{TEM}-positive isolates on chicken eggshell surfaces sold in traditional markets in Surabaya. Because the PCR products were not sequenced and phenotypic ESBL confirmation was not performed, the results do not confirm a specific bla_{TEM} variant or an ESBL-producing phenotype. Although these results do not establish eggs as major reservoirs of AMR or demonstrate farm-to-consumer transmission, they identify eggshell surfaces as a potential interface for exposure to antimicrobial-resistant bacteria and resistance determinants. Continued surveillance combined with improved hygiene, responsible antimicrobial use, and measures to prevent cross-contamination is warranted within a One Health framework.

DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

GENERATIVE AI DECLARATION

Generative AI tools were used to assist with language editing and manuscript preparation. The authors reviewed and revised the content and take full responsibility for the accuracy and integrity of the manuscript.

AUTHORS' CONTRIBUTIONS

AAS, LRS, and PK: Conceptualized and designed the study and critically reviewed the manuscript. MHE and WT: Conducted the study, analyzed the data, and critically reviewed the manuscript. DAAK, SMY, RZA, IBM, and JYHT: Analyzed and interpreted the data and critically reviewed the manuscript. ARK and BPP: Analyzed and interpreted the findings, drafted the manuscript, and led its revision through the final version. SR and M'AK: Interpreted the findings and critically revised the manuscript. All authors reviewed and approved the final manuscript and agreed to be accountable for the work.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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