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Isolation and Identification of Antibiotic-Resistance Pathogenic Bacteria from Chicken Eggshells

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ABSTRACT

Chicken eggs represent a globally important source of animal protein for human consumption. Chicken eggs can serve as a source of bacterial contamination, including pathogenic bacteria resistant to antibiotics. Improper handling during purchase, storage, and cooking may allow pathogenic bacteria to infect consumers. The present study aimed to identify antibiotic-resistant pathogenic bacteria in chicken eggshells. This preliminary and exploratory study was carried out in several stages. These stages included collecting ten chicken eggs from five different stores in Semarang, Indonesia, performing bacterial culture, testing susceptibility to novobiocin and amoxicillin via the Kirby-Bauer method, performing Gram staining, and examining colony and cell morphology. The next stage included DNA extraction, 16S rRNA gene amplification, sequencing, and confirmation using morphological observations. The current results indicated that seven out of ten egg samples contained bacteria in their shells. Molecular identification revealed that bacterial isolates labeled A, B, C, D, E, F1, F2, G1, and G2, isolated from chicken egg, were closely related to *Staphylococcus durrellii*, *Staphylococcus schleiferi*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Staphylococcus shinii*, *Staphylococcus saprophyticus*, *Staphylococcus cohnii*, and *Pseudomonas ficuserectae*, respectively. Antibiotic resistance results demonstrated that samples A, B, C, F1, F2, and G2 were resistant to amoxicillin, while samples C and F2 were resistant to novobiocin. Chicken eggshells were found to include antibiotic-resistant pathogenic bacteria, mainly *Staphylococcus* and *Streptococcus* species, often resistant to amoxicillin. These current results indicated possible public health risks and highlight the importance of enhancing food safety measures.

Keywords: Chicken eggshell, Contamination, Antibiotic-resistant bacteria

INTRODUCTION

The increase in infections from antibiotic-resistant bacteria has emerged as a significant global public health issue. Worldwide, foodborne diseases affect one in every ten people, with approximately 600 million cases and 420,000 deaths reported worldwide (Solís et al., 2023). Resistance to antimicrobial agents undermines treatment success and is associated with increased morbidity, mortality, and economic impacts (Hinson et al., 2025). Food products, particularly those of animal origin such as chicken eggs, have been recognized as potential reservoirs of antibiotic-resistant pathogenic bacteria (Nhung et al., 2017; Mak et al., 2022). Over the past several decades, global egg production and consumption have shown a substantial upward trend, increasing from approximately 14 million tons in 1961 to more than 1.600 million tons in 2021 (Solís et al., 2023).

During production, packaging, and distribution, eggshell surfaces can become contaminated through exposure to environmental sources, fecal matter, and human handling, increasing the risk of cross-contamination (Abou-Jaoudeh et al., 2024). Several studies have reported the presence of pathogenic bacteria,

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including *Escherichia coli* and *Salmonella* spp. (Wahyuningsih, 2019), and *Staphylococcus aureus* (Thaha et al., 2024), on eggshell, with some isolates demonstrating resistance to multiple antimicrobial agents (Kousar et al., 2021; Abou-Jaoudeh et al., 2024). Bacteria on the eggshell surface can penetrate the egg and contaminate its contents. Bacterial penetration into eggs is caused by poor shell quality. *Pseudomonas* and *Salmonella* species are known to penetrate eggs effectively (De Reu et al., 2006). The age of the chicken and the characteristics of the eggshell, such as its surface area, thickness, and number of pores, affect the ability of bacteria to penetrate the egg (Whiley and Ross, 2015). Bacterial transfer is affected by handling during washing, environmental humidity, and storage conditions (Whiley and Ross, 2015).

Eggshell contamination can cause food poisoning if eggs are not properly cooked, and many consumers are unaware of this risk. Eggs sold in markets and supermarkets often still have animal fecal matter attached to their shells. Consumers frequently handle eggs without gloves and neglect handwashing afterward, increasing the risk of contamination. Pathogenic bacteria isolated from eggs have been confirmed to be antibiotic-resistant and zoonotic (Dafale et al., 2020). Antibiotic-resistant bacteria in poultry products typically originate from contaminated feed that contains antibiotic residues or resistant microorganisms. Using antibiotics in animal feed can cause selective pressure, promoting the survival and proliferation of resistant bacteria in the poultry's gastrointestinal tract (Sana et al., 2025). This may subsequently contaminate eggs and poultry-derived products. *Salmonella* strains with antimicrobial resistance have been found in eggs and egg-derived products. These strains included multidrug-resistant (MDR) variants that show decreased susceptibility to β-lactam antibiotics, fluoroquinolones, and aminoglycosides (Castro-Vargas et al., 2020; Abreu et al., 2023). The MDR bacteria not only increase virulence but also complicate clinical management of infections.

Identification of bacterial species and resistance profiles from chicken eggshells is essential to reduce the risk of foodborne disease transmission. The 16S rRNA gene is widely used for bacterial species identification because it contains conserved and hypervariable regions, which allow differentiation among bacterial taxa (Frank et al., 2008). The present study aimed to isolate and identify antibiotic-resistant pathogenic bacteria from chicken eggshells using conventional microbiological techniques and molecular techniques based on the 16S rRNA gene.

MATERIALS AND METHODS

Ethical approval

The entire sample and research methods have been approved by the Health Research Ethics Committee, Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang, with ethical clearance number 236/KE/12/2025.

Sampling and bacterial isolation

A total of ten chicken eggs were randomly collected from five stores in the Kedungmundu area, Semarang, Indonesia. This location was selected because it was surrounded by three hospitals, elementary schools, universities, and densely populated residential areas. Chicken eggs were randomly collected from those available for sale at the markets during the sampling time. Eggshells were washed with sterile 0.9% physiological NaCl, and the wash solution was collected in sterile containers. One mL of the wash solution was mixed with 9 mL of sterile 0.9% NaCl and homogenized using a vortex mixer. Serial dilutions (10^1-10^5) were prepared, and 1 mL of each dilution was inoculated onto blood agar plates (BAP; Turista et al., 2019). The plates were incubated at 37°C for 24 hours. Colony growth and hemolytic activity were observed on the following day. Colonies from all chicken eggshells were subcultured on blood agar plates (Oxoid, United Kingdom) and nutrient agar (NA; Merck, Germany) to obtain pure isolates.

Gram staining

Pure colonies were subjected to Gram staining. Colonies were smeared onto glass slides in a circular area of approximately 2-3 cm in diameter and heat-fixed by passing the slide through a flame three times. The smears were stained with crystal violet for three minutes, rinsed with running water, and treated with Lugol's iodine for two minutes. After rinsing, decolorization was performed with alcohol until the smear became clear, then rinsed with water (Smith and Hussey, 2005). The smear was counterstained with fuchsin for one minute, rinsed, air-dried, and examined under a light microscope (Leica Microsystems, Germany) at 100× magnification using immersion oil. Bacterial detection relied on microscopic examination of the Gram reaction and cell morphology, especially identifying Gram-positive cocci in clusters or similar arrangements in chains.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the Kirby-Bauer disc diffusion method on Mueller-Hinton

agar (MHA) with an agar depth of approximately 4 mm, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Pure colonies were suspended in sterile 0.9% NaCl solution, and the turbidity was adjusted to match a 0.5 McFarland standard (Hudzicki, 2009). One ml of the suspension was spread evenly on agar plates using sterile cotton swabs. Amoxicillin discs were then placed on the agar surface and incubated at 37°C for 18-24 hours. Novobiocin and amoxicillin were selected because they are commonly used antibiotics in Indonesia (Limato et al., 2022; Sari et al., 2025). The concentrations of novobiocin and amoxicillin were each 25 µg. After incubation, the diameters of inhibition zones were measured using calipers, and the susceptibility profiles were interpreted according to the CLSI guidelines (Gaur et al., 2023).

Bacterial subculture and suspension preparation

Pure bacterial colonies from stock cultures were inoculated into brain heart infusion (BHI) broth (Oxoid, United Kingdom) using sterile inoculation loops. The cultures were incubated at 35-37°C for 18-24 hours to promote bacterial growth. Bacterial growth was confirmed by visual observation of broth turbidity. The resulting cultures, including all colonies from eggshells, were then used for genomic DNA extraction, following an established bacterial cultivation protocol (Wijesinghe et al., 2019).

Bacterial genomic DNA extraction

Bacterial suspensions obtained from each sample were first centrifuged at 6000 rpm for 15 minutes, after which the supernatant was carefully removed. The pellets of Gram-positive bacteria were resuspended in 480 µL of 50 mM EDTA, followed by the addition of 120 µL lysozyme, and incubated at 37°C for 45 minutes to facilitate cell wall disruption. The samples were then centrifuged at 12,000 rpm for two minutes, and the supernatant was discarded.

Pellets from both Gram-positive and Gram-negative bacteria were subsequently mixed with 750 µL of lysis buffer and vortexed. Protein digestion was carried out by adding 20 µL of proteinase K (10 mg/mL) and mixing for 15 minutes. The samples were incubated at 55°C for 30 minutes, with intermittent vortexing every 10 minutes to enhance cell lysis and protein degradation.

Following incubation, the samples were centrifuged at 3000 rpm for 15 minutes to separate cellular debris, and the resulting supernatant was transferred into new microcentrifuge tubes. Phenol (700 µL) was then added, and the samples were gently mixed for 30 minutes. The samples were centrifuged at 12,000 rpm for 10 minutes, and the aqueous layer was carefully transferred to a new microtube. By adding an equal volume of cold absolute ethanol and gently mixing, the DNA was precipitated. The samples were centrifuged at 12,000 rpm for 10 minutes, after which the supernatant was discarded. The resulting DNA pellets were washed three times with 500 µL of ethanol 70%, followed by centrifugation at 12,000 rpm for 10 minutes after each wash (Kartika et al., 2021). Finally, the pellet was air-dried and resuspended in 200 µL TE buffer. The DNA concentration and purity were assessed using a MaestroNano pro spectrophotometer (Maestrogen, GMI, USA).

Genomic DNA electrophoresis and PCR products

An agarose gel 1% was prepared by dissolving 0.3 g of agarose powder in 30 mL of 0.5× Tris-Borate-EDTA (TBE) buffer following established laboratory protocols (Kartika et al., 2021). The solution was heated in a microwave until the agarose was fully dissolved and became clear. GoldView (ELK Biotechnology CO., Ltd, USA) was added at a volume of 3 µL and mixed gently to avoid bubble formation. Once the agarose gel had completely solidified, the comb was gently removed, and the gel was positioned in an electrophoresis tank containing 0.5× TBE buffer until fully covered. The DNA samples were mixed with ExcelDye™ 6X DNA loading dye, Tri-color (SMOBIO Technology, Inc., Taiwan) before loading into the gel wells. Electrophoresis was performed at a constant voltage of 100 V for 30-45 minutes. After electrophoresis, DNA bands stained with GoldView™ nucleic acid stain (Beijing SBS Genetech Co., Ltd., China) were visualized and documented using a UV transilluminator (Lee et al., 2012).

Amplification of the 16S rRNA gene

Purified DNA from bacterial contaminants isolated from eggshells served as a template for amplification of the 16S rRNA gene (Meutia et al., 2022). The PCR amplification was performed in a total reaction volume of 25 µL using PowerPol 2X PCR Mix with dye (ABClonal, Massachusetts, USA). Each reaction contained 1 µL of the universal bacterial forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1 µL of the reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′; Frank et al., 2008), along with genomic DNA as the template and nuclease-free water to adjust the final volume. Thermal cycling was initiated with a hot-start activation step at 95°C for three minutes, followed by

denaturation at 95°C for 30 seconds, primer annealing at 56°C for 30 seconds, and extension at 72°C for two minutes. A final cooling step was carried out at 4°C with 35 cycles (Kartika et al., 2021). The amplified PCR products were subsequently submitted for sequencing, following established protocols for 16S rRNA gene analysis (Abellan-Schneyder et al., 2021). The study procedure, starting from bacterial culture isolated from eggshells, Gram staining, antibiotic resistance testing, DNA extraction, PCR, and sequencing, was summarized in Figure 1, which was created in BioRender (BioRender Inc., Canada).

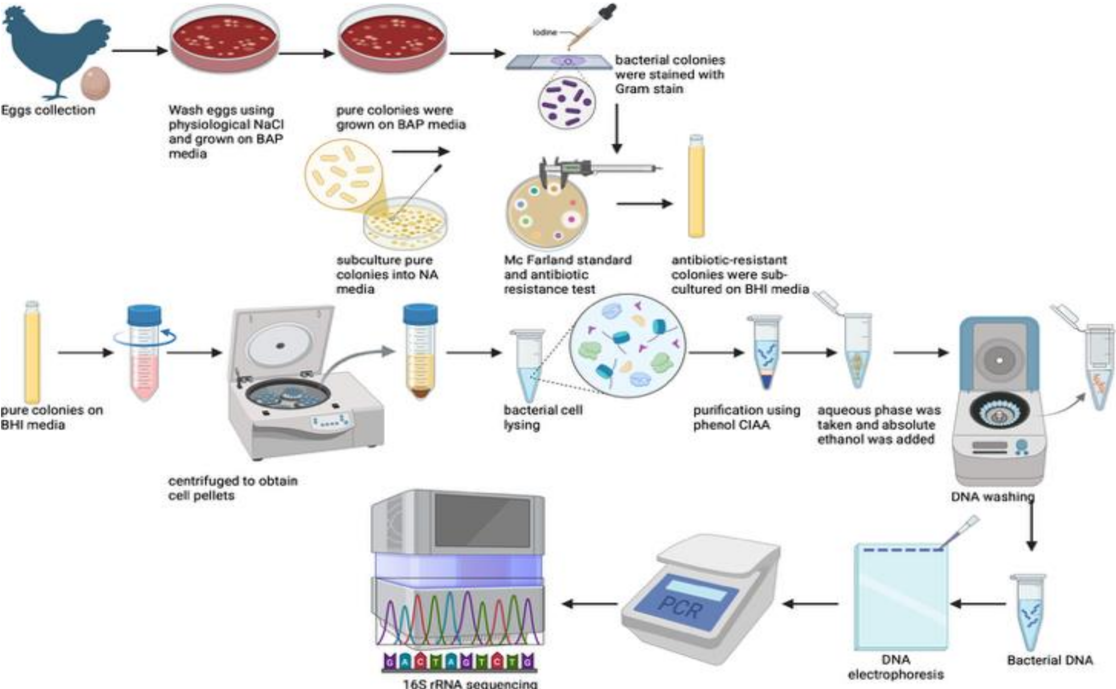

Figure 1. The identification of pathogenic bacteria from chicken eggshells. Created in BioRender; Kartika et al. (2021)

RESULTS

Identification of bacterial colonies

Colonies were assessed for the presence or absence of clear or discolored zones around the growth area following incubation in BAP. The absence of color changes (hemolysis zone) around the bacterial colonies A, B, E, F1, F2, G1, and G2 indicated the presence of γ-hemolysis bacteria (Figure 2). Analysis of bacterial colonies on BAP demonstrated that colonies A, B, D, E, F2, and G1 were white, colonies F1 and G2 were yellow, and colony C was creamy (Table 1). Bacterial colonies were categorized based on distinct colony morphology, including size, shape, margin, elevation, pigmentation, and hemolytic patterns on BAP.

Most bacterial colonies isolated from eggshells exhibited a round shape, smooth consistency, entire margin, and convex elevation (Table 1). Only two colonies, C and D, exhibited a flat elevation and shared other morphological features, including hemolytic activity on erythrocytes on BAP (Table 1).

Bacterial identification using Gram staining

Additional differentiation was performed using Gram staining to assess cell morphology and Gram reaction. Bacteria isolated from the surface of eggshells were identified as Gram-positive, indicated by their purple color (Figure 3). All isolates were Gram-positive bacteria, as evidenced by purple color after the staining process (Merck, Germany).

Antibiotic resistance was determined based on the diameter of the inhibition zone obtained from the disc diffusion assay and classified according to the CLSI interpretive criteria. Resistance to amoxicillin and novobiocin was tested in all bacterial isolates obtained from the outer surface of eggshells. Antibiotic susceptibility testing revealed resistance to amoxicillin in bacterial isolates A, B, C, F1, F2, and G2, as determined by the disc diffusion method (Figure 4). Resistance to novobiocin was observed in isolates C and F2 (Figure 4).

The PCR amplification of the 16S rRNA gene produced amplicons of approximately 1500 bp. All samples exhibited a single DNA band aligned at ~1500 bp, although band intensity varied. This variation was due to differences in the DNA concentration used as the PCR template. Fainter bands indicated lower concentrations of bacterial DNA in the PCR mix, while thicker bands reflected higher concentrations (Figure 5).

All PCR products of the 16S rRNA gene were sequenced using Sanger sequencing. The sequencing results confirmed that the bacterial species found on chicken eggshells were consistent with cell morphology identified by Gram staining and hemolysis patterns. Samples C and D showed β-hemolysis and were molecularly identified as *Streptococcus pyogenes* and *Streptococcus agalactiae*, respectively. Gram staining showed that samples A, B, E, F1, F2, G1, and G2 contained *Staphylococcal* arrangements, aligning with sequencing results that classified them within the genus *Staphylococcus*. In contrast, samples C and D exhibited *Streptococcal* arrangements, aligning with molecular identification as genus *Streptococcus* (Table 2).

Molecular identification using the 16S rRNA gene revealed that several species contaminating eggshells were pathogenic and thus carried zoonotic potential, including sample C, which was identified as *Streptococcus pyogenes* (Zondervan et al., 2021), and sample E as *Staphylococcus aureus* (Zondervan et al., 2021; Mlynarczyk-Bonikowska and Rudnicka, 2025; Table 2).

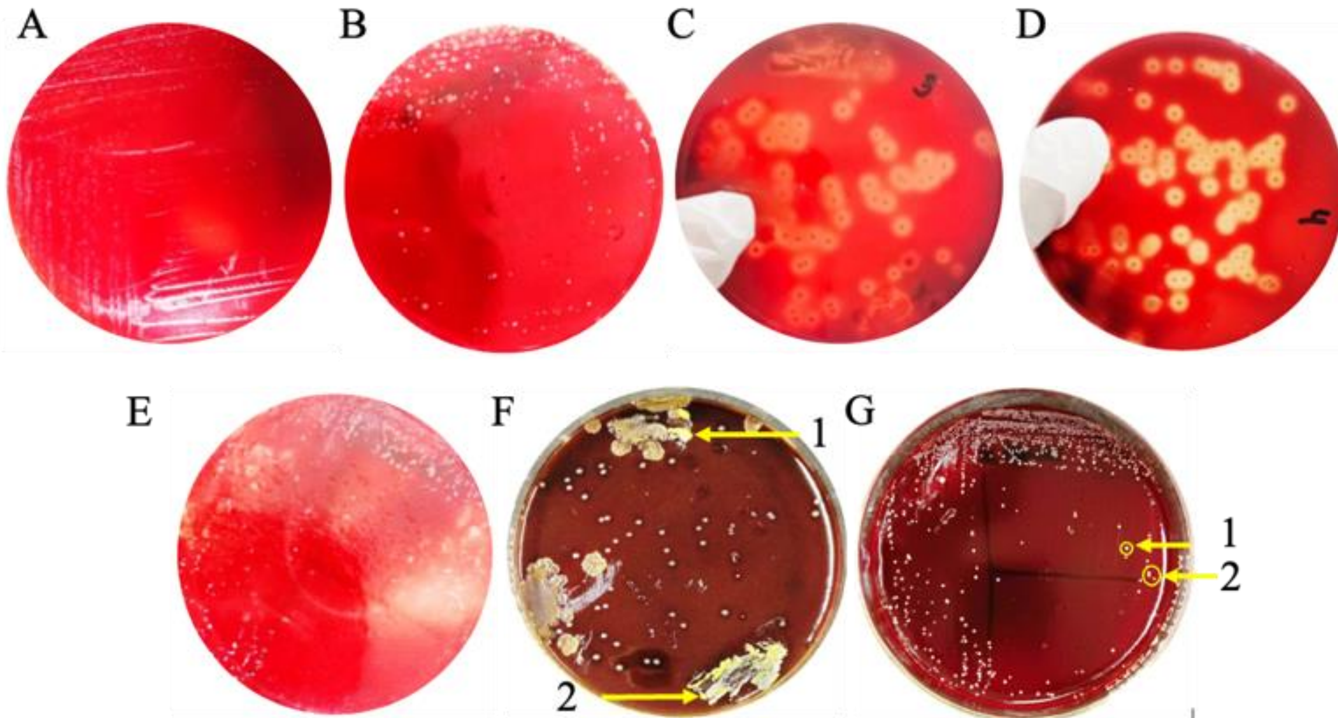

Figure 2. Hemolytic activity of bacterial isolates on blood agar plates of seven egg samples. **A, B, E,** and **F1, F2, G1, G2**: γ-hemolysis, **C** and **D**: β-hemolysis

Table 1. Identification of bacterial colonies on the blood agar plate isolated from chicken eggshells, Semarang, Indonesia

Sample	Colony color	Colony form	Size (mm)	Colony consistence	Edge of the colony	Elevation of the colony	Type of hemolysin
A	White	Round	1	Smooth	Entire	Convex	γ-Hemolysis
B	White	Round	1	Smooth	Entire	Convex	γ-Hemolysis
C	Cream	Round	0.5	Smooth	Entire	Flat	β-Hemolysis
D	White	Round	0.5	Smooth	Entire	Flat	β-Hemolysis
E	White	Round	1	smooth	Entire	convex	γ-Hemolysis
F1	Yellow	Round		Smooth	Entire	Convex	γ-hemolysis
F2	White	Round	1	Smooth	Entire	Convex	γ-hemolysis
G1	White	Round	1	Smooth	Entire	Convex	γ-hemolysis
G2	Yellow	Round	0.8	smooth	Entire	Convex	γ-hemolysis

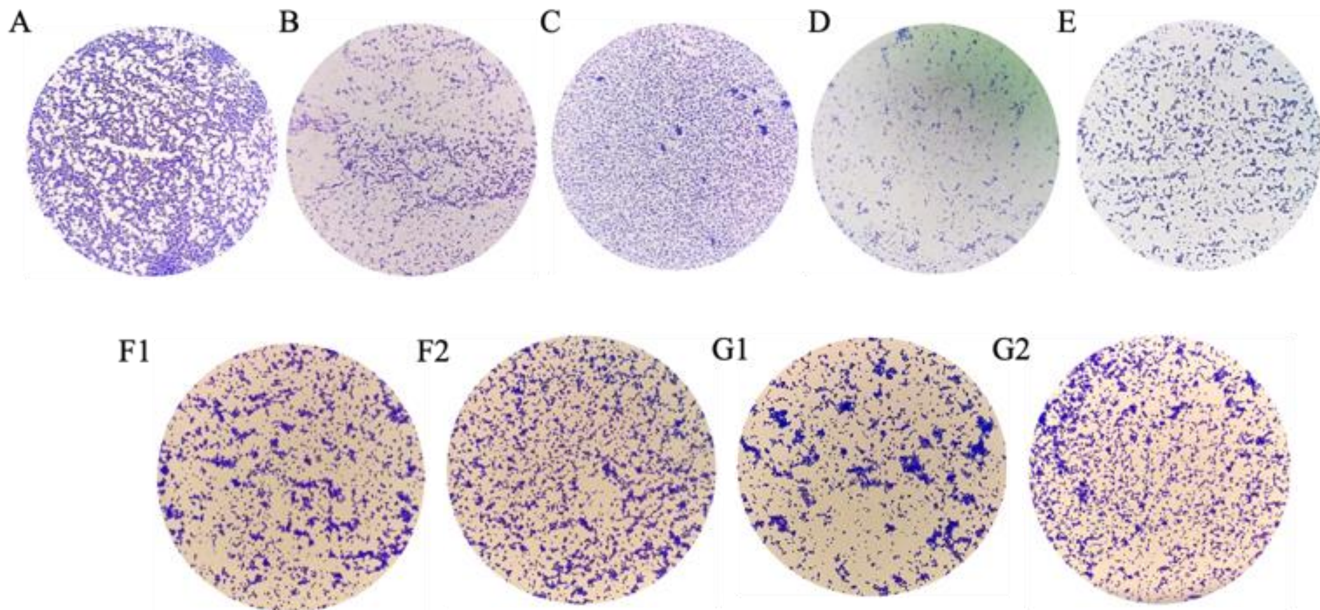

Figure 3. Identification of isolates of bacteria using Gram staining (100× magnification). Isolates were recovered from seven eggs. Letters **(A-G)** denote the origin of eggs. Subscript numbers (1-2) denote different isolates from the same egg

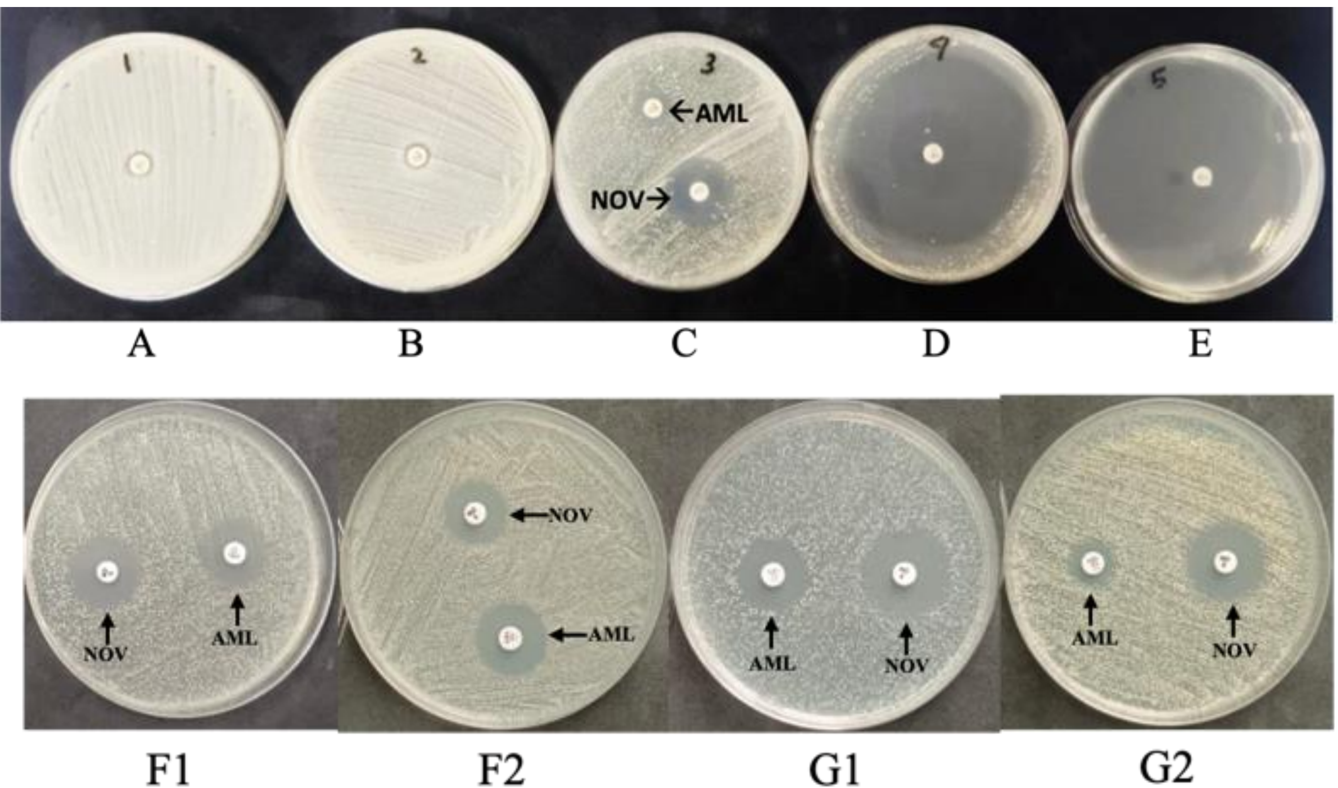

Figure 4. Antibiotic resistance test using the Kirby-Bauer method on Mueller-Hinton agar. **A, B, D**, and **E** were tested only with amoxicillin. **C**, **F1, F2, G1**, and **G2** were tested with novobiocin and amoxicillin.

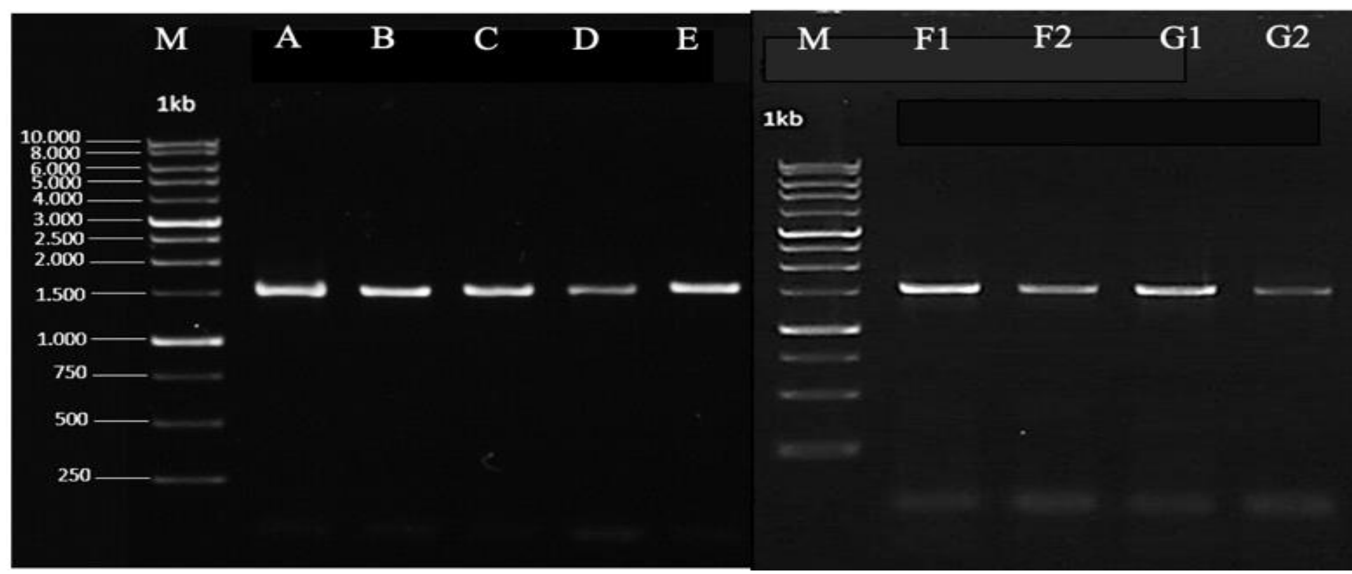

Figure 5. Amplification of the 16S rRNA gene in bacterial isolates from chicken eggshells. The DNA band size of all samples was approximately 1500 bp. M: Marker, **A-G2**: Isolates bacteria from chicken eggshells

Table 2. Characteristics of bacteria based on Gram staining, antibiotic resistance testing, and molecular identification using the 16S rRNA gene

Sample	Cell color	Cell form	Cell arrangement	Gram type	Antibiotic drug resistance		Identification based on 16S rRNA
					Amoxicillin	Novobiocin	
A	Purple	Coccus	Staphylococci	+	R	-	*Staphylococcus durrellii* strain 27_4_6_LY (NR_181502.1)
B	Purple	Coccus	Staphylococci	+	R	-	*Staphylococcus schleiferi* (NR_037009.1)
C	Purple	Coccus	Streptococci	+	R	R	*Streptococcus pyogenes* strain JCM 5674 (NR_112088.1)
D	Purple	Coccus	Streptococci	+	S	-	*Streptococcus agalactiae* ATCC 13813 strain JCM 5671 (NR_113262.1)
E	Purple	Coccus	Staphylococci	+	S	-	*Staphylococcus aureus* strain S33 R (NR_037007.2)
F1	Purple	Coccus	Staphylococci	+	R	S	*Staphylococcus shinii* strain Ga1-10 chromosome, complete genome. CP150685.1.
F2	Purple	Coccus	Staphylococci	+	R	R	*Staphylococcus saprophyticus* strain RCB148 16S ribosomal RNA gene,partial sequence. KT260360.1.
G1	Purple	Coccus	Staphylococci	+	S	S	*Staphylococcus cohnii* strain Tr15B 16S ribosomal RNA gene, partial sequence. MH210861.1.
G2	Purple	Coccus	Staphylococci	+	R	S	*Pseudomonas ficuserectae* strain Ps42 16S ribosomal RNA gene, partial sequence. KU750786.1

Gram type: + Means Gram-positive, R: Antibiotic resistance, S: Sensitive to antibiotics. A dash mark (-) shows that the test was not conducted.

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DISCUSSION

The present results indicated that eggshells can serve as a reservoir for potentially pathogenic and antibiotic-resistant bacteria, even in eggs sold through modern retail outlets in Semarang, Indonesia. Similar observations have been reported in previous studies, which documented the presence of pathogenic bacteria on eggshells collected from both traditional markets and stores (Wahyuningsih, 2019; Abebe et al., 2020). The detection of hemolytic activity and antibiotic resistance in the current isolates aligns with reports indicating that eggshells are a major pathway for foodborne bacterial transmission (Harage and Al-Aqaby, 2024).

Antibiotic-resistant isolates were identified during the present study, reinforcing concerns about the role of poultry products in the spread of antimicrobial-resistant bacteria. Improper handling and storage after purchase can promote bacterial survival on the eggshell's surface and raise the risk of bacteria entering the egg's interior, as reported in other studies.

Although there are many packaged egg options, such as omega chicken eggs, cage-free eggs, and regular eggs, packaged chicken eggs are more expensive. Packaged chicken eggs are cleaner and have no feces attached to their outer shells compared to eggs sold individually. However, people prefer to buy eggs individually because they are cheaper and more flexible in terms of the number purchased. Dirty eggshells are more likely to carry harmful bacteria. Improper storage at home can allow bacteria from the shell to enter the egg and contaminate it.

Blood agar plate media is differential because it can show hemolysis patterns (α, β, and γ). Bacteria capable of lysing erythrocytes are important virulence factors often associated with pathogens. These bacteria can produce hemolysin, an enzyme that can lyse erythrocytes either partially or completely (Amaria et al., 2023). Bacteria with γ-hemolysis do not lyse red blood cells. β-hemolytic bacteria are characterized by a clear/transparent zone around the colony on BAP (Buxton, 2005). Bacteria isolated from chicken eggshells were cultured on BAP to assess their capacity to induce erythrocytes. A clear zone was observed on the medium from the complete lysis of erythrocytes, leading to hemoglobin degradation and a loss of coloration. β-hemolytic activity is typical of group A Streptococci, such as *Streptococcus pyogenes*, and group B Streptococci, such as *Streptococcus agalactiae*, both of which are important human pathogens.

Bacterial pigments, metabolic products, and interactions with blood components in the growth medium influence colony color. White colonies are generally produced by Gram-positive cocci, which lack pigments and therefore appear transparent to milky white. Yellow colonies on BAP usually stem from bacteria that produce carotenoid or flavone pigments, which act as antioxidants and protect the bacteria from oxidative stress. Cream-colored colonies often indicate bacteria that make slight or no pigment (Liu and Nizet, 2009).

Colony identification on BAP was based on colony color, shape, size, consistency, margin, and elevation, which helps identify pure colonies and differentiate them from possible contaminants. Gram staining was used to differentiate bacteria based on the thickness of the peptidoglycan layer (Smith and Hussey, 2005). Information on the peptidoglycan structure is clinically important, as it supports appropriate antibiotic application in patients (Nikolaidis et al., 2014).

Antibiotic-resistant bacteria can contaminate eggshells through poultry antibiotics or decontamination efforts, since decontamination cannot eradicate all bacteria, it may inadvertently promote resistance in the survivors (Vale et al., 2024). Bacteria develop resistance to amoxicillin through several mechanisms. In Gram-negative bacteria, resistance is mediated by producing metallo-β-lactamases with broad activity. β-lactamase inhibitors such as sulbactam and clavulanic acid are commonly used to restore amoxicillin activity, particularly against organisms producing β-lactamases (Huang and Zhou, 2025). These inhibitors bind irreversibly to the catalytic site of bacterial β-lactamases, contributing to resistance (Tehrani and Martin, 2018). Other β-lactam resistance mechanisms include the production of β-lactamases, plasmid-mediated transfer of AmpC enzymes, extended-spectrum β-lactamases (ESBLs) such as blaTEM, blaSHV, blaCTXM, and carbapenemases. In Gram-positive bacteria, resistance occurs through mutations in penicillin-binding proteins (PBPs) and reduced expression of outer membrane proteins (OprD), thereby reducing drug penetration (Sodhi et al., 2021). Bacterial isolates resistant to amoxicillin were identified in samples A, B, C, F1, F2, and G2 during the present study.

Novobiocin was also tested due to its relevance to antibiotic resistance in pathogenic bacteria reported in Indonesia. Species of the genus *Aeromonas* causing septicemia were identified in Sukabumi, Jepara, and Surabaya, Indonesia, and were resistant to novobiocin (Kusdarwati et al., 2018). Novobiocin, a natural

aminocoumarin antibiotic derived from *Streptomyces*, has been used for decades. Novobiocin inhibits bacterial DNA gyrase and blocks ATP binding (Rodríguez-Cerrato et al., 2010). Novobiocin is particularly effective against *Staphylococci*, including MRSA, and other Gram-positive bacteria (Gombert and Aulicino, 1984). Bacterial isolates resistant to novobiocin were identified in samples C and F2 during the present study.

Based on the current results, nine bacterial isolates with distinct morphologies were found in seven eggs, and molecular identification was performed. Nine colonies were obtained from eggshells; just six colonies were found to be resistant to the antibiotic amoxicillin, and three samples were sensitive. Previous studies have indicated that chicken eggshells may be contaminated with *E. coli, Enterobacter* spp.*, Enterococcus* spp.*, Klebsiella* spp.*, Citrobacter* spp.*, Pseudomonas* spp.*, S. aureus,* and fungi such as *Aspergillus* spp.*, Penicillium* spp.*, Cladosporium* spp.*, Fusarium, Rhizopus* spp.*,* and *Mucor* spp. (Mansour et al., 2015).

Amoxicillin belongs to the β-lactam class of antibiotics and exhibits activity against a wide range of Gram-positive and Gram-negative bacteria. The European Respiratory Society has confirmed amoxicillin as a preferred initial treatment option for community-acquired lower respiratory tract infections (Malhotra-Kumar et al., 2016). Amoxicillin was selected for its widespread use as a first-line antibiotic for community-acquired infections and its broad application in both human and veterinary medicine (Huttner et al., 2020). Amoxicillin, either as a single agent or in combination with clavulanic acid, is widely used in European countries (Sodhi et al., 2021). As a broad-spectrum antibiotic, amoxicillin inhibits peptidoglycan polymerization, an essential component of bacterial cell walls.

Based on molecular testing during the current experiment, Sample A was similar to *Staphylococcus durrellii*. *Staphylococcus durrellii* has the potential to be an opportunistic pathogen and is found in different environments, including poultry and human skin. Sample A was Gram-positive and resistant to amoxicillin. Amoxicillin-resistant bacteria complicate the treatment of zoonotic infections, particularly those transmitted via food products such as eggs (Kerek et al., 2025). Meanwhile, sample B exhibited similarities with the *Staphylococcus schleiferi*. *Staphylococcus schleiferi* is commonly found on the skin and mucous membranes of dogs, but can also be an opportunistic pathogen in humans (Sewid and Kania, 2024). Sample B was Gram-positive and resistant to amoxicillin. The potential resistance of the sample B isolate to amoxicillin reinforces the importance of microbiological monitoring of poultry products such as eggshells (Farrukh et al., 2025).

Sample C was similar to the *Streptococcus pyogenes*. *Streptococcus pyogenes* is an important human pathogen, causing infections such as pharyngitis, fever, and skin infections (Kilsgård et al., 2016). *Streptococcus pyogenes* is β-hemolytic and belongs to the group A Streptococcus (GAS) and has specific virulence factors, such as M protein and streptolysin (SLS/SLO), which play major roles in adhesion, immune evasion, and tissue damage (Thacharodi et al., 2025). In addition to molecular analysis, the similarity between sample C and *Streptococcus pyogenes* was also confirmed by morphological characteristics.

Sample D was similar to the *Streptococcus agalactiae* strain JCM 5674 (NR_113262.1). *Streptococcus agalactiae* is the primary cause of neonatal infections such as sepsis, pneumonia, and meningitis, and can infect adults, especially pregnant women and immunocompromised individuals (Carvalho et al., 2025). The virulence factors of *Streptococcus agalactiae* include polysaccharide capsules and invasive enzymes such as hemolysin and hyaluronidase (Wang et al., 2014). Culture-based (phenotypic) and molecular (genotypic) methods for detecting *Streptococcus agalactiae* demonstrated high consistency and relevance in assessing food safety and zoonotic risk (da Rocha et al., 2020).

Sample E was similar to *Staphylococcus aureus* strain S33 R (NR_037007.2). *Staphylococcus aureus* was found on the surface of chicken and quail eggs and was resistant to β-lactam antibiotics, including oxacillin, and tested positive for the *nuc* and *mecA* genes (Pondit et al., 2018). Previous studies in Indonesia have detected *Staphylococcus aureus* in fresh and processed poultry egg products (Thaha et al., 2024). Contamination levels were higher in raw eggs, reaching approximately 1×10^2 colonies/g, whereas lower bacterial counts, around 1×10^1 colonies/g, were observed in processed eggs, such as salted eggs. Moreover, MRSA has been found in egg-related samples in Indonesia (Thaha et al., 2024). Additionally, *Staphylococcus aureus* was also found to be resistant to amoxicillin in patients from the Pakem Health Center, Yogyakarta, Indonesia (Estiningsih et al., 2023).

Sample F1 was similar to the *Staphylococcus shinii* strain Ga1-10 chromosome (CP150685.1). *Staphylococcus shinii* is a non-aureus *Staphylococcus* found in poultry house bioaerosols (Ghaffar et al., 2025). The poultry house environment is the typical habitat of non-aureus *Staphylococcus.* In general, *Staphylococcus* is found on

the skin and mucous membranes of poultry. There are seven species of non-aureus *Staphylococcus*, namely *Staphylococcus equorum, shinii, pseudoxylosus, cohnii, gallinarum*, that have been confirmed and identified using whole genome sequencing (Ghaffar et al., 2025). *Staphylococcus* can be coagulase-negative or coagulase-positive, but both can cause serious infections. *Staphylococcus shinii* is a new species that is closely related to *Staphylococcus pseudoxylosus* (Cho et al., 2022). Contamination with *Staphylococcus shinii* has been observed in fermented meat, and the associated microbiota influences the meat's color and aroma (Sosa-Fajardo et al., 2024). The present study indicated that sample F1 was resistant to amoxicillin. The current results were consistent with previous studies, indicating that some bacteria from poultry-house layer barn bioaerosol samples were MDR-resistant (Ghaffar et al., 2025).

Based on 16S rRNA gene analysis, sample F2 was similar to *Staphylococcus saprophyticus* strain RCB148 (KT260360.1). *Staphylococcus saprophyticus* has virulence factors that are crucial for evaluating the risk of food-related human diseases caused by cross-contamination of eggs (Ge et al., 2016). Previous studies indicated that *Staphylococcus saprophyticus* was among the identified bacteria capable of causing urinary tract infections in mice when injected intraperitoneally at 10^7 CFU (Kline et al., 2010). *Staphylococcus saprophyticus* is a common cause of urinary tract infection in immunocompromised patients, with a reported incidence of 10-20% (Widerström et al., 2012). The present study indicated that sample F2 was resistant to amoxicillin and novobiocin. Previous studies have reported that *Staphylococcus* spp. isolated from chicken eggshells were resistant to penicillin but remained susceptible to vancomycin (Kadhim et al., 2020). It has been reported that the surface of chicken eggshells can harbor antibiotic-resistant bacteria, including *Staphylococcus* spp. and *Staphylococcus aureus*, potentially posing a risk of transmission to humans (Pondit et al., 2018).

Sample G1 was similar to the *Staphylococcus cohnii* strain Tr15B 16S ribosomal RNA gene (MH210861.1). *Staphylococcus* sp. is a common bacterium often found in chicken intestines, both pathogenic and nonpathogenic forms. The mecA gene has been detected in *Staphylococcus* sp. from domestic chickens, while the *ermC*, *tetK*, and *tetM* genes were found in *Staphylococcus* isolated from domestic and other poultry groups. *Staphylococcus saprophyticus and Staphylococcus cohnii* were identified in poultry intestines and exhibited antibiotic resistance (Syed et al., 2020).

Sample G2 was similar to the *Pseudomonas ficuserectae* strain Ps42 16S ribosomal RNA gene, partial sequence (KU750786.1). *Pseudomonas* sp. is pathogenic mainly to humans and can be transmitted to humans through contamination from animals or consumption of animal-derived products. *Pseudomonas* sp. can contaminate chicken eggs, originating from chicken feces or the surrounding environment (Abd El-Ghany, 2021). *Pseudomonas* sp. can form biofilms that act as a protective layer, enabling it to survive on egg surfaces (Liu et al., 2023). Biofilms are also produced to protect *Pseudomonas* sp. from antibiotic penetration and facilitate persistent, immune-resistant chronic infections (Sharma et al., 2023). The handling of chicken eggs, from purchase at the market to storage, should be carefully monitored. Eggs should be processed or cooked before consumption (Al-Ashmawy et al., 2013).

The present study had several limitations. Antibiotic susceptibility testing was limited to two antibiotics, amoxicillin and novobiocin, which could not fully represent the broader antimicrobial resistance profiles of bacteria isolated from eggshells. The selection of these antibiotics was based on their frequent use as first-line or screening agents and their relevance in clinical and agricultural settings.

CONCLUSION

The present study demonstrated that chicken eggshells may harbor Gram-positive, pathogenic, antibiotic-resistant bacteria. The integration of phenotypic assays and 16S rRNA-based identification confirmed the presence of *Staphylococcus* and *Streptococcus* species. Based on the current findings, contamination by antibiotic-resistant pathogenic bacteria posed a zoonotic risk. Egg contamination with bacteria resistant to beta-lactam antibiotics posed a challenge for the clinical treatment of zoonotic infections. Egg contamination can be anticipated by monitoring the egg supply chain, from farms and distribution to markets. Simple washing or handling is not sufficient to eliminate pathogenic bacteria. The present study highlighted the importance of educating the public on thoroughly cooking eggs and promoting proper handling practices among traders and consumers. Nevertheless, future studies should include a broader range of antibiotics from different classes, such as fluoroquinolones, aminoglycosides, and tetracyclines, to provide a more comprehensive assessment of resistance patterns.

DECLARATIONS

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Aprilia Indra Kartika contributed to the study's conceptualization, developed the methodology, performed data validation, drafted the original manuscript, and acquired research funding. Febe Marchia Charanita contributed to data validation, conducted the investigation, and participated in drafting the original manuscript. Meutia Srikandi Fitria contributed to the conceptualization of the study and managed project administration.

Sri Darmawati contributed to the conceptualization and formal analysis of the study, oversaw project administration, and supervised the research activities. Anousin Homsana contributed to the supervision of the study and was responsible for funding acquisition

All authors read and approved the last edition of the manuscript for publication in the present journal.

Competing interests

There is no conflict of interest regarding the present study and manuscript.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors. The authors did not use AI in writing the article.

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