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Antimicrobial Resistance of *Enterobacteriaceae* Isolates from Chicken Eggs Sold at Bukidnon, Philippines

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Abstract

The isolation of *Enterobacteriaceae* from chicken eggs and their antimicrobial resistance was investigated. The study utilized various selective, differential, and biochemical media for the isolation and identification of the organisms. The Kirby-Bauer disk diffusion method was then used to assess resistance to four commonly used antimicrobials. Results were interpreted based on reference values set by the Clinical Laboratory Standards Institute. A total of 101 *Enterobacteriaceae* organisms, including *Citrobacter* spp., *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Salmonella* spp., *Serratia* spp., and *Shigella* spp., were identified from eggshell surfaces and egg contents. In decreasing order of the number of resistant isolates, the bacterial isolates showed resistance to ampicillin, followed by trimethoprim-sulfamethoxazole, ciprofloxacin, and doxycycline. Multidrug resistance patterns were observed, with numerous representative bacterial isolates showing resistance to all antimicrobials. The findings highlight a public health concern and underscore the need for enhanced monitoring of antimicrobial-resistant bacteria in food sources, the judicious use of antimicrobials in poultry layer production, improved hygiene practices in food production and handling, and recommendations for further research to better understand resistance mechanisms.

Keywords

Antibiotic resistance, food safety, multidrug-resistant bacteria, One Health, table egg

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Introduction

Eggs and egg products, which are primary sources of protein and amino acids, are often linked to food poisoning outbreaks (De Reu, 2006). Bacteria may contaminate eggs on their surface or their egg contents. The shell surface of eggs may be contaminated through contact with polluted surfaces (soil, chicken cages, packaging, handling). Bacteria on the surface of the egg can also pass through the cuticle, into the eggshell pores, then through eggshell membranes, and move into the internal egg contents (Smith *et al*., 2000; Musgrove *et al*., 2004). Various microorganisms contaminate eggs, increasing the risk of developing foodborne infectious diseases. The outer eggshell, in particular, harbors several microbial organisms, including *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus* spp., *Bacillus* spp., and *Listeria monocytogenes* (Mahdavi *et al*., 2012).

The family *Enterobacteriaceae* comprises opportunistic members that are associated with various infections. Normal intestinal microbiota are the most common isolates of *Salmonella* spp., *E. coli*, *Shigella*, *Klebsiella* spp., *Enterobacter* spp., and *Serratia* spp. They are recognized as opportunistic pathogens, associated with infections such as acute gastroenteritis, food poisoning, diarrheal diseases, and enteric fever (Mahon and Lehman, 2022).

An additional concern regarding foodborne bacteria is their resistance to antimicrobial agents. The use of antimicrobials in food and agriculture is linked to the development of antimicrobial resistance in bacteria. In the Philippines, the lack of enforcement of antimicrobial prescriptions for farmers, the accessibility of antimicrobials in local agriculture-veterinary (agrovet) supply outlets, and a lack of awareness about the use of antimicrobials may accelerate the occurrence and development of antimicrobial resistance (Barroga *et al*., 2020).

With the statements at hand, the study aimed to investigate the prevailing *Enterobacteriaceae* bacteria in samples from chicken eggs purchased from two public markets in Bukidnon and evaluate the sensitivity of isolated bacteria against different antimicrobials. The study's results provide necessary information for monitoring this local public health concern.

Materials and Methods

The study was conducted in two central public markets in Bukidnon, Philippines, over six weeks from April to May 2024. A total of 120 chicken eggs were collected. In each market, two chicken eggs were collected from five randomly selected stalls weekly for six weeks.

Sample Collection and Processing

One hundred twenty white and intact (without visible deformities and cracks) chicken eggs, regardless of source, soiling, size, and time of purchase, were randomly collected. The samples were collected weekly and processed immediately upon collection. The chicken eggs were placed in sterile bags using sterile gloves and transported to the College of Veterinary Medicine Microbiology Research Laboratory for sample processing and examination. The chicken eggs were tested within 24 hours after collection. Bacterial isolation was done separately using the eggshell surface and egg contents, following the procedure outlined by Zhao Ge *et al.* (2016) with minor modifications. For the eggshell surface, a sterile cotton swab dipped in normal

saline solution (NSS) was swabbed around the entire shell surface, then immersed in a tube containing 10 mL of nutrient broth. The tube was incubated at 36°C for 24 hours. For the egg contents, the entire surface of the chicken egg was disinfected using a gauze pad wetted with 70% ethanol, and the chicken egg was allowed to dry for 90 seconds before being broken into a sterile flask to avoid contamination. The egg contents were mixed thoroughly, and 5 mL of egg homogenate was aspirated. The aspirate was then inoculated into 45 mL of nutrient broth and incubated at 36°C for 24 hours.

Bacterial Isolation

After enrichment in a nutrient broth, a loopful of cultured broth was Gram-stained to identify Gram-negative and Gram-positive bacteria. The cultured broth was streaked into the selective media, namely the MacConkey agar, Eosin Methylene Blue agar, and Salmonella-Shigella agar. The inoculated selective media were then incubated at 36°C for 24 hours. Colonies from the agar plates were subjected to biochemical tests, including the oxidase test, indole test, methyl red test, Voges-Proskauer test, citrate test, motility test, urease test, and triple sugar iron slant (Cheesbrough, 2006). The results were then used to identify bacterial isolates based on published characteristics (Farmer *et al.*, 2010).

Antimicrobial Susceptibility Testing

Four antimicrobial discs, namely ciprofloxacin (5 µg), ampicillin (10 µg), doxycycline (30 µg), and trimethoprim-sulfamethoxazole (1.25/23.75 µg), were commercially acquired (Oxoid™, Thermo Fisher Scientific, UK) and used to test the antimicrobial susceptibility of the bacterial isolates by the Kirby-Bauer disk diffusion method, as described by Islam *et al.* (2018) with minor modifications. After the desired bacterial suspension was prepared, a loopful of the bacterial suspension was added to 9 mL of sterile distilled water, and the process was continued until a turbidity comparable to a 0.5 McFarland standard was achieved. A sterile swab was then dipped into the resulting bacterial suspension and streaked onto a Mueller-Hinton Agar Plate, ensuring that the bacterial suspension covered all areas of the plate. Four antimicrobial discs, one for each of the previously mentioned antimicrobials, were then placed into the inoculated Mueller-Hinton Agar, ensuring that each disc had enough space from the other to avoid overlapping inhibition zones. After the discs had been placed, the plates were covered, inverted, and incubated at 36°C for 24 hours. The resulting zones of inhibition were measured using a calibrated caliper and interpreted according to the recommendations of the Clinical Laboratory Standards Institute (Weinstein, 2019), categorized as Susceptible (S), Intermediate (I), and Resistant (R).

Results

The identity and occurrence of *Enterobacteriaceae* isolates in chicken eggs are summarized in Table 1. A total of 101 bacterial isolates were identified. The representative results of bacterial isolation and identification are shown in Figure 1. The prevalent bacteria isolated were *Klebsiella* spp. (19.81%) and *Enterobacter* spp. (30.69%). *Salmonella* spp. (14.85%), *Proteus* spp. (9.90%), *Serratia* spp. (8.91%), *Shigella* spp. (6.93%), *Escherichia coli* (4.95%), and *Citrobacter* spp. (3.96%) were also isolated from the samples.

Table 1 also summarizes the occurrence of *Enterobacteriaceae* on the eggshell surface and egg content. A higher bacterial contamination was detected on eggshell surfaces (69/101,

68.32%) than in the egg contents (32/101, 31.68%).

Table 1. Identity and percentage of bacteria isolated from eggshell surfaces and egg contents.

Isolated Bacteria	Sample type				Total	
	Eggshell surface		Egg contents			
	No. of isolated bacteria	%	No. of isolated bacteria	%	No. of isolated bacteria	%
Citrobacter spp.	2	1.98	2	1.98	4	3.96
Escherichia coli	3	2.97	2	1.98	5	4.95
Enterobacter spp.	22	21.79	9	8.90	31	30.69
Klebsiella spp.	11	10.89	9	8.92	20	19.81
Proteus spp.	7	6.93	3	2.97	10	9.90
Salmonella spp.	14	13.86	1	0.99	15	14.85
Serratia spp.	7	6.93	2	1.98	9	8.91
Shigella spp.	3	2.97	4	3.96	7	6.93
Total	**69**	**68.32**	**32**	**31.68**	**101**	**100.00**

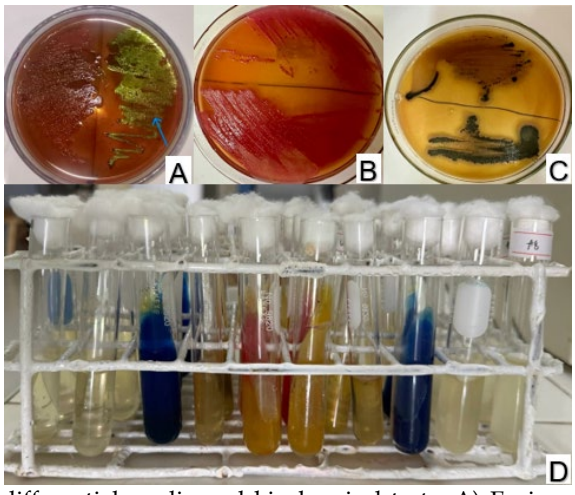

Figure 1. Representative results of differential media and biochemical tests: A) Eosin methylene blue (EMB) agar showing characteristic green metallic sheen (blue arrow), indicating the presence of *Escherichia coli*; B) MacConkey agar showing characteristic pink to red colonies, indicating the presence of lactose-fermenting Enterobacteriaceae; C) Salmonella-Shigella agar (SSA) showing colorless colonies with black centers, indicating the presence of hydrogen sulfide-producing *Salmonella* spp.; D) biochemical tests shown from left to right: methyl-red (MR) medium (-), Voges-Proskauer (VP) medium (-), sodium citrate slant (+), sulfur indole motility (SIM) medium (-), triple sugar iron (TSI) slant (alkali/acid), second TSI slant (alkali/acid), second SIM tube (weak H2S production), second sodium citrate slant (+), second VP medium (-), and second MR medium (-).

The antimicrobial sensitivity profiles of the bacteria isolated from eggs are summarized in Table 2. Representative results of antimicrobial sensitivity testing are shown in Figure 2. All of the identified 101 *Enterobacteriaceae* isolates showed resistance to at least one subject antimicrobial: ciprofloxacin, doxycycline, ampicillin, and trimethoprim-sulfamethoxazole. These antimicrobials were chosen as they are commonly used in the local poultry layer industry and are relevant to public health. Most bacterial isolates (91/101, 90.10%) were resistant to ampicillin.

Furthermore, a high number of resistant bacterial isolates against trimethoprim-sulfamethoxazole (79/101, 78.22%) were reported, while many bacterial isolates are also resistant to ciprofloxacin (45/101, 44.55%). Lastly, the least degree of bacterial resistance is observed with doxycycline (39/101, 38.61%). The *Enterobacteriaceae* isolates from the egg samples presented multidrug resistance, indicating resistance to two or more antimicrobials.

Table 2. Antimicrobial sensitivity profiles of Enterobacteriaceae isolated from eggs using four commonly used antimicrobials.

Isolates	No. of tested isolates	Antimicrobial discs											
		Ciprofloxacin (5 µg)			Ampicillin (10 µg)			Doxycycline (30 µg)			TMP-SMZ (1.25/23.75 µg)		
		S	I	R	S	I	R	S	I	R	S	I	R
Citrobacter spp.	4	0	0	4	0	0	4	0	0	4	0	1	3
Escherichia coli	5	3	1	1	0	0	5	2	2	1	1	0	4
Enterobacter spp.	31	12	8	11	1	4	26	12	8	11	4	3	24
Klebsiella spp.	20	7	0	13	1	0	19	9	2	9	3	1	16
Proteus spp.	10	4	1	5	1	0	9	3	3	4	1	2	7
Salmonella spp.	15	4	6	5	0	0	15	5	4	6	1	1	13
Serratia spp.	9	4	3	2	0	2	7	4	2	3	1	2	6
Shigella spp.	7	1	2	4	0	1	6	2	4	1	0	1	6
Total	**101**	**35**	**21**	**45**	**3**	**7**	**91**	**37**	**25**	**39**	**11**	**11**	**79**

Legend: S = Susceptible; I = Intermediate resistance; R = Resistant

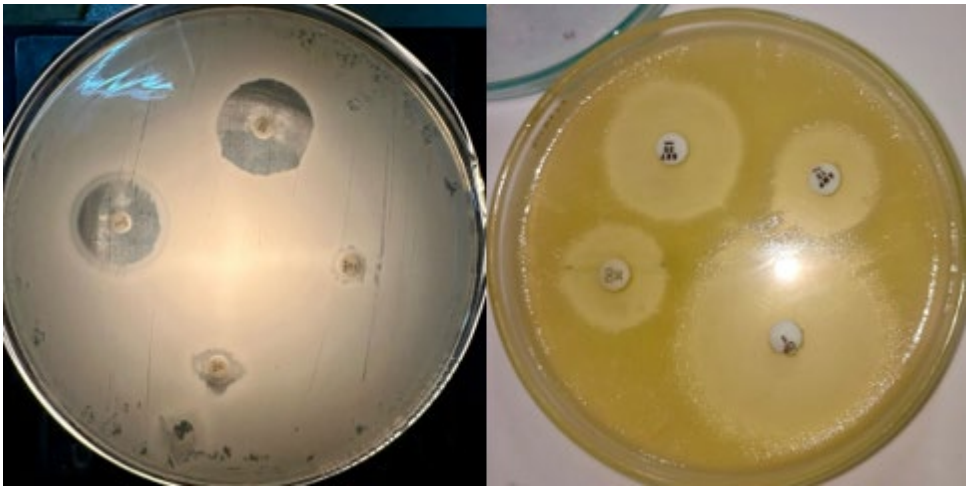

Figure 2. Representative results of antimicrobial sensitivity test using the Kirby-Bauer disk diffusion method conducted on Mueller-Hinton agar plates: Left: bacterial isolate shows resistance (≤13 mm) to ampicillin (10 µg) and resistance (≤10 mm) to trimethoprim.

Discussion

The use of complete randomization in selecting chicken eggs significantly enhanced the methodological quality of this study. Randomly selecting chicken eggs from stalls in public markets minimized selection bias, thereby improving the accuracy of the reported findings on the prevalence of bacteria isolated and their antimicrobial resistance profiles. The results of bacteria isolated in this study were similar to those of Musgrove *et al.* (2008), who isolated *Proteus* spp., *Enterobacter* spp., and *Klebsiella* spp. from unwashed and commercially washed eggs. In the study by Amer *et al.* (2013), 44 Enterobacteriaceae isolates were obtained from commercial eggs, comprising *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Salmonella* spp., *Shigella* spp., *Serratia* spp., and *Escherichia coli*. Important factors that influence the bacterial contamination of chicken eggs (both in egg shells and egg contents) are the freshness of eggs (Stepień-Pyśniak, 2010; Amer *et al.*, 2013), temperature and duration of storage (Theron *et al.*, 2003; Cook *et al.*, 2005; Stepień-Pyśniak, 2010), environment of storage, transport conditions, presence of aerosolized feces in eggshell surface (Theron *et al.*, 2003, Kilonzo-Nthenge *et al.*, 2016), eggshell translucency (Chousalkar *et al.*, 2010) handling of eggs (Kilonzo-Nthenge *et al.*, 2016), and inadequacy of packaging (Wales *et al.*, 2022). These conditions were observed during chicken egg collection, wherein eggs were often stacked in trays, one on top of the other, in a place bustling with people. This may imply concerns about the freshness of the eggs, storage conditions, and environmental factors at the time of sale. Exposing eggs to room temperature for an extended period will cause faster deterioration; thus, as soon as eggs are released from the farm and sold in public markets, immediate storage in colder temperatures (16-17 °C) should be practiced, along with 80% relative humidity for safe consumption of eggs for up to 7 days from the time of purchase (Feddern *et al.*, 2017).

A higher number of bacteria was isolated from the eggshell surface, which agrees with the study by Jambalang *et al.* (2017). This

higher presence increases the likelihood of the bacteria penetrating the shell and contaminating the egg's internal contents (Smith *et al*., 2000). In eggshell surface samples, *Enterobacter* spp. and *Klebsiella* spp. were the predominant bacteria isolated, in agreement with the results of Stepień-Pyśniak (2010), who found that *Enterobacter* spp. and *Klebsiella* spp. were the dominant isolates in eggshell samples. Other isolated bacteria in eggshell surface samples included *Proteus* spp., *Serratia* spp., *Salmonella* spp., *Shigella* spp., *Citrobacter* spp., and *Escherichia coli*. Kilonzo-Nthenge *et al.* (2016) also reported that the eggshell surface samples were positive for *E. coli*, *Enterobacter* spp., *Serratia* spp., *Citrobacter braakii*, and *Klebsiella* spp. *Salmonella* spp. was isolated only from the eggshell surfaces, indicating that not all eggs with contaminated shells had their internal contents contaminated with the same species of bacteria. The dominance and predilection of bacteria on the eggshell surface are attributed to two factors. Bacteria such as *Enterobacter* spp. and other members of the *Enterobacteriaceae* family can produce biofilms (Bai *et al*., 2021), which serve as a protective layer for survival in harsh environments (Kathi, 2024). Other bacteria, such as Klebsiella spp., isolated from the eggshell surface, are also able to survive due to their thick cell walls (Kowalczyk *et al*., 2022), which enable them to survive for extended periods and be carried in poultry products (Gong *et al.,* 2023).

On the other hand, the bacteria isolated in the egg contents were *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *E. coli*, *Proteus* spp., *Salmonella* spp., *Shigella* spp., and *Serratia* spp. *Escherichia coli* is noted to be present in egg contents, as it was on the eggshell surface, similar to the findings. Kilonzo-Nthenge *et al.* (2016) reported that egg content samples were positive for bacteria, similar to those isolated in this study. This indicates that although the eggshell surface may be contaminated by a myriad of *Enterobacteriaceae*, the cuticle of the eggshell provides a protective function that prevents contamination of egg contents. Bacterial contamination may occur in eggs with a compromised cuticle, allowing flagellated bacteria, such as *Salmonella typhimurium* and *Escherichia coli*, to penetrate the eggshell through its pores (Al-Bahry *et al*., 2012). The results underscore the importance of maintaining rigorous hygiene practices in egg handling to minimize the risk of contamination, including regular cleaning of laying areas, thorough washing and sanitizing of eggshells, and ensuring that eggs are cooked to a safe temperature before consumption (De Reu, 2006; Musgrove *et al*., 2008).

The emergence and spread of antimicrobial resistance can be attributed to several factors. To understand this, it is essential to emphasize that the antimicrobials used in this study were selected as indicators of antimicrobial resistance due to their widespread use in the local poultry layer industry. One of the most significant factors contributing to the emergence and spread of antimicrobial resistance is the misuse and overuse of antimicrobials by the industry. Due to the need to maintain high egg production, these antimicrobials are used as the first-line treatment for flock sickness and as part of prophylaxis and growth promotion. These practices significantly contribute to antimicrobial resistance (Marshall and Levy, 2011), and the prolonged use of these antimicrobials in the poultry industry may lead to the development of antimicrobial resistance over time (Okorie-Kanu *et al*., 2016).

In the Philippines, the lack of enforcement of antimicrobial prescriptions for farmers, the accessibility of antimicrobials to farmers through local agriculture-veterinary (agrovet) supply outlets, and a lack of awareness about the use of antimicrobials may accelerate the occurrence and development of antimicrobial resistance (Barroga *et al*., 2020).

Conclusion

The study identified eight genera of *Enterobacteriaceae* bacteria from egg samples collected from two central public markets in Bukidnon. Contamination rates were significantly higher on the eggshell surface than in the egg contents, indicating a greater likelihood of isolating bacteria from surface swabs. All isolated bacteria exhibited resistance to at least one of the antimicrobials tested, with multidrug resistance being prevalent. Resistance rates were exceptionally high for ampicillin and trimethoprim-sulfamethoxazole, while some isolates showed susceptibility to ciprofloxacin and doxycycline. These findings underscore the urgent need for targeted antimicrobial therapy based on specific resistance profiles, as the rapid emergence and spread of antimicrobial-resistant bacteria pose significant threats to veterinary and medical practices, as well as public health. A multifaceted approach is therefore recommended to address this issue, which includes regular surveillance of antimicrobial resistance in poultry production, promoting judicious use of antimicrobials, improving farm hygiene and biosecurity practices, considering vaccination and the use of probiotics as replacements to antimicrobial use, and educating laypeople and relevant stakeholders on the dangers caused by antimicrobial resistance.

Approval of Ethical Commission

Written permission from the local government units of the study sites was obtained and approved before the study's conduct. The study also complied with the requirements for obtaining a biosafety permit from the University Biosafety and Biosecurity Committee of Central Mindanao University. This ensured that sample processing, bacterial isolation, and antimicrobial sensitivity testing were conducted in accordance with appropriate biosafety considerations.

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Author's Contribution

PJSS: Conceptualization, methodology, data curation, and writing (review and editing). RPST and JBMCT: Methodology, investigation, analysis, and writing (original draft). VBJ, MLBM, and JMO: Conceptualization and writing (review and editing).

Conflict of Interest

The authors declare no conflict of interest that may influence the work reported in this manuscript.

Data Availability Statement

The data gathered and used for analysis in this study are available upon reasonable request from the corresponding author.

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