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Antimicrobial Susceptibility Pattern of Bacterial Isolates from Raw and Frozen Chicken Meat

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ABSTRACT

Quality control in meat is essential, as it carries the risk of different pathogenic bacteria affecting human health. The study aimed to evaluate raw and frozen chicken meat samples quantitatively and qualitatively. Five raw and 5 frozen chicken meat samples were collected from retail shops in Kathmandu valley to detect the presence of *E. coli, Salmonella* spp., *Pseudomonas* and *Staphylococcus aureus*. The isolates were also examined for multidrug resistance (MDR) and phenotype detection of methicillin-resistant *Staphylococcus aureus* (MRSA). The total plate count for raw chicken was too high in all the samples, while that of frozen chicken meat was 2.3 x 10^6 cfu/ml. The total coliform count and total fecal coliform count ranged from 5.7 x 10^4 to 4.7 x 107 cfu/ml, and 1.01 x 10^2 to 1.12 x 10^2 cfu/ml, respectively. Among different isolates, *Escherichia coli* was detected in all 10 samples and most of the isolates (i.e. 70 %) were found to be resistant to the antibiotics ciprofloxacin. Similarly, *Salmonella* was present in 8 out of 10 (80%) samples, where 37.5% of isolates were found to be resistant toward azithromycin and ceftazidime. *Staphylococcus aureus* was identified in 6 samples, with all isolates showing resistance to ampicillin and cefoxitin (100%). Out of a total of 24 bacteria isolated, 7 (29.16%) exhibited multi-drug resistance; one isolate was *E. coli* and the remaining were *Staphylococcus aureus*. The results of this study showed the presence of potential human pathogens. These findings emphasize the critical need to enhance quality control and quality assurance systems in meat shops across the Kathmandu valley, ensuring public health safety.

Keywords: Raw meat; Frozen meat; *Escherichia coli; Salmonella; Staphylococcus aureus*; Multidrug-resistant (MDR)

Introduction

Chicken meat is a nutrient-rich food essential for growth and metabolic functions in living beings. Its consumption has consistently risen globally due to its affordability and reputation as an economical protein source in diets. In Nepal, the daily requirement for poultry meat is around 150,000 kilograms, while the annual production of broiler chickens reported by the NHIA (Yadav et al., 2020) falls within the range of 25 to 30 million. A variety of normal flora communities reside in different parts of chickens' bodies, such as the skin, feet, feathers, feces, GI tract, and visceral cavity.

The acceptable total viable count for chicken meat ranges between 10^4 and 10^6 (Adhikari et al., 2019). While meat from healthy animals generally poses minimal microbial risk, stress or infection can increase microbial loads (Thanigaivel, 2015). Contaminating sources include external and internal animal parts such as hair, hide, hooves, and the gastrointestinal tract, where microbial colonization is common. Slaughtering, transportation, butcher hygiene, equipment, blades, and water used in processing also contribute to contamination (Bantawa et al., 2018). Additionally, processed meat may introduce microorganisms through additives, tools, and the processing environment. Applying proper slaughtering methods and food safety practices helps reduce microbial presence in fresh meat (Wardhana et al., 2021).

E. coli, *Salmonella*, *S. aureus*, *Campylobacter*, and *Listeria* are pathogenic microorganisms that are commonly found in poultry and poultry products that pose potential health risks. *E. coli* is recognized as a commensal microbe. It generally resides in the digestive system of warm-blooded mammals. Although *E. coli* is regarded as a non-pathogenic organism and part of the normal flora, some strains have the potential to cause various illnesses in humans (Worku et al., 2022). *E. coli* strains are

Gram-negative facultative anaerobes that can induce diarrheal illness when they become pathogenic (Sowmya et al., 2014). Even though *E. coli* is frequently found in the gut and is classified as a fecal coliform, it is important to remember that chicken meat and organs contain *E. coli* (Ranasinghe et al., 2022). Consuming contaminated poultry products is a common route of infection for humans. Pathogenic *E. coli* has become a paramount health concern (Dave, 2011).

Salmonella is commonly found in dairy products and raw poultry and is a major cause of foodborne illnesses worldwide. Being the primary pathogen responsible for bacterial food poisoning, *Salmonella* poses significant public health risks, especially through handling or consuming undercooked poultry (Gautam et al., 2019).

Pseudomonas, a psychrotrophic bacterium capable of growing below 7 °C, is a key spoilage agent in chilled meat. Species like *P. fluorescens, P. fragi, P. lundensis, P. migulae,* and *P. putida* are frequently found in chilled meat (Fessler et al., 2011; Fresno et al., 2013). In poultry, *Pseudomonas* infections spread quickly and cause high mortality, with *P. aeruginosa* particularly linked to deaths in day-old chicks and embryos (Hassan et al., 2018).

Staphylococcus, part of the normal skin flora in humans and animals, often colonizes areas like the axillae and nasal canal (Klurfeld, 2018). *Staphylococcus* has been found in eggs and household chickens, with isolated strains showing antibiotic resistance (Odwar et al., 2014). This is especially concerning in poultry products due to risks posed by MRSA strains, which produce staphylococcal enterotoxins leading to foodborne illness. Cross-contamination can occur during processing, particularly if MRSA-infected handlers practice poor hygiene (Saud et al., 2019*).*

Antibiotics are drugs designed to combat bacterial infections by inhibiting or killing bacteria. However, their overuse and misuse have led to the rise of antibiotic-resistant bacteria, which can transfer to humans through contaminated meat. Proper cooking practices help eliminate foodborne and antibiotic-resistant bacteria, while consuming undercooked or raw meat increases the risk of severe infections (Madhup et al., 2021). The emergence of antimicrobial resistance (AMR) has reduced antibiotic effectiveness, posing major challenges in medicine (Maharjan et al., 2019). Methicillin-resistant *Staphylococcus aureus* (MRSA), developed by mutations in penicillin-binding proteins, is a significant global concern (Shrestha et al., 2017). Misuse of antibiotics has greatly contributed to the spread of multidrug-resistant (MDR) bacteria and MRSA, threatening both human and animal health. This study aimed to investigate microbiological contamination in raw and frozen chicken meat from retail shops in Kathmandu Valley and to assess the misuse of antibiotics in poultry farming through antibiotic resistance testing.

Materials and methods

Sample size

A total of ten chicken meat samples were collected from three different districts of Kathmandu Valley. Out of 10 samples, five were raw chicken meat and five were vacuum-packed frozen chicken meat.

Sample collection and processing

About 50 grams of chicken meat samples were collected in clean, dry ziploc bags and transported in an ice box to the laboratory of St. Xavier's College for microbiological analysis. A laboratory-based descriptive study was conducted from December 2022 to June 2023.

Quantitative bacteriological analysis

Total viable count

Ten grams of weighted and minced chicken meat sample was subjected to 90 ml of 0.85 % saline water. Serial dilution was performed up to 10^{-5}. One ml from 10^{-3} and 10^{-5} dilutions was pour-plated on PCA (plate count agar) and incubated for 24 hours at 37 °C.

Total coliform count and total fecal coliform count

One mL of chicken meat sample from 10^{-3} and 10^{-5} dilutions was poured into VRBA (violet red bile agar). Over-layering of the plate was done by the addition of 5 ml of VRBA to prevent surface growth

and spreading of colonies. The plates were incubated at 37 °C for total coliform and 44.5 °C for fecal coliform for 24 hours (Parvin et al., 2020).

Qualitative bacteriological analysis
Enrichment
25 grams of the weighted and minced chicken meat samples were subjected to 225 ml of 1% peptone water (1:10 w/v). It was then incubated at 37 °C for 24 hours in a shaker incubator.

Isolation and identification of E. coli
A loopful of culture suspension from pre-enrichment was inoculated by quadrant streaking on MacConkey agar (MA), which was incubated at 37 °C for 24 hours. After incubation, the pink- colored colonies observed were further confirmed by Gram's staining and biochemical test.

Isolation and identification of Salmonella species
One ml of chicken meat sample from pre-enrichment was transferred to 9 ml selenite F broth and incubated at 37 ˚C for 24 hours. After 24 hours of incubation, a sample from selenite F broth was streaked on *Salmonella-Shigella* agar (SS) plates. The plate was incubated for the next 24 hours at 37 ˚C. After incubation, black-centered colonies of *Salmonella* were further confirmed by Gram's staining and biochemical tests.

Isolation and identification of Staphylococcus aureus
A loopful of culture suspension from peptone water was streaked on mannitol salt agar (MSA) agar and incubated at 37 °C for 24 hours. After incubation, the yellow colonies observed were further confirmed by Gram's staining and biochemical tests.

Isolation and identification of Pseudomonas species
A loopful of culture suspension from peptone water was taken and streaked on cetrimide agar medium (HiMedia) supplemented with glycerol and evenly spread. The inoculated plates were incubated at 37 °C for 48 hours. After incubation, greenish colonies were observed and further confirmed by Gram's staining and biochemical test (Anihouvi et al., 2024).

Antibiotic susceptibility tests
Antibiotic susceptibility tests were performed on Muller-Hinton agar (MHA) following the modified Kirby-Bauer disc diffusion method. The organisms inoculated in the nutrient broth were compared to a 0.5 McFarland standard turbidity tube. A sterile swab was dipped into adjusted inoculum, and the carpet culture was performed on Muller-Hinton agar. The selected antibiotics for the identified organisms were placed on MHA, which was then incubated at 37 °C for 24 hours. The zone of inhibition was measured, and the results were evaluated as resistant (R), sensitive (S), and intermediate (I). The organism that showed resistance to three or more groups of antibiotics was termed multidrug-resistant (MDR). All the microbiological activities in this study adhered to CLSI guidelines (CLSI 2020).

Screening of MRSA
All the identified *Staphylococcus* isolates were subjected to phenotypic detection of MRSA by the disk diffusion method using cefoxitin (30 µg). The pure isolates of *Staphylococcus aureus* were transferred to nutrient broth, followed by incubation at 37 ˚C for 24 hours. The prepared inoculum was matched with a 0.5 McFarland standard turbidity tube. Then, sterile cotton swabs were dipped in the inoculum, and carpet culture was performed on MHA plates. The cefoxitin (30 µg) disc was placed on the inoculated MHA plates and incubated at 37 ˚C for 24 hours. After incubation, MRSA was reported to have a diameter of zone of inhibition of less than or equal to 21 mm (CLSI 2020).

Result

Total plate count of raw and frozen chicken meat
The comprehensive analysis of total plate count for 5 distinct raw and frozen chicken meat samples procured from different locations has been presented in Table 1. The microbial count in all the raw chicken meat samples was found to be too many to count.

Similarly, in frozen chicken, only sample F3 exhibited a microbial count of 2.3 x 10^6 cfu/ml, while no detectable microbial count was observed in the remaining samples.

Table 1: Total plate count of raw and frozen chicken meat

Samples	Average cfu/ml	Samples	Average cfu/ml
R1	TMTC	F1	ND
R2	TMTC	F2	ND
R3	TMTC	F3	2.3 x 10^6
R4	TMTC	F4	ND
R5	TMTC	F5	ND

* ND = Not detected * R= Raw chicken meat * F= Frozen chicken meat * TMTC = Too many to count

TCC and TFCC of raw and frozen chicken meat in VRBA

The total coliform count (TCC) and total fecal coliform count (TFCC) of raw and frozen chicken meat samples are shown in Table 2, where all raw samples had detectable TCC ranging from 5.7 × 10^4 cfu/ml to 4.7 × 10^5 cfu/ml. Still, TFCC was absent in samples R1, R3, and R5, while samples R2 and R4 showed TFCC values of 1.12 × 10^2 cfu/ml and 1.01 × 10^2 cfu/ml, respectively. Among the five frozen samples, only sample F3 showed both TCC and TFCC, with values of 2.1 × 10^5 cfu/ml and 1.1 × 10^2 cfu/ml, respectively.

Table 2: TCC and TFCC of raw and frozen chicken meat

Samples	Average cfu/ml		Samples	Average cfu/ml	
	37 ˚C	45 ˚C		37 ˚C	45 ˚C
R1	5.9 x 10^4	ND	F1	ND	ND
R2	5.7 x 10^4	1.12 x 10^2	F2	ND	ND
R3	1.12 x 10^5	ND	F3	2.1 x 10^5	1.1 x 10^2
R4	4.7 x 10^5	1.01 x 10^2	F4	ND	ND
R5	1.79 x 10^5	ND	F5	ND	ND

* NG = Not detected * R= Raw chicken meat * F= Frozen chicken meat * TMTC = Too Many To Count

Percentage of bacteria isolated from raw and frozen chicken meat

During the process of isolating bacteria from ten samples of chicken meat, *E. coli* was isolated from all 10 samples, representing a 100% occurrence. On the other hand, *Salmonella* was found in eight out of ten samples, accounting for an 80% occurrence. *Staphylococcus* was detected in six out of ten samples, indicating a 60% occurrence.

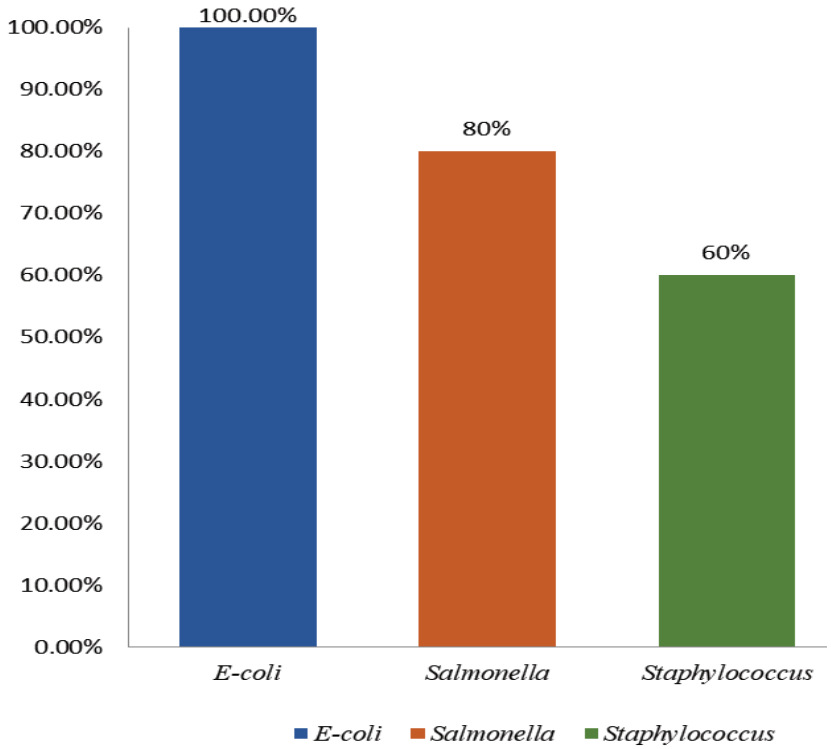

Figure 1: Bacterial isolates from chicken meat

Antibiotic susceptibility testing of E. coli

E. coli was isolated from all of the provided samples, indicating a prevalence of 100%. The susceptibility patterns of the isolated *E. coli* colonies obtained from chicken meat are shown in table 3. In this study, a total of five antibiotics were utilized for susceptibility testing, and the results revealed notable resistance rates. 70% of *E. coli* isolates displayed a resistance pattern against Ciprofloxacin, while 40% of *E. coli* isolates demonstrated a resistance pattern against Ampicillin. Similarly, 30% of isolates exhibited resistance patterns against cefotaxime and tetracycline. However, none of the isolates showed any resistance against nitrofurantoin. Among the 10 samples analyzed, only one raw chicken meat sample exhibited multidrug resistance.

Table 3: Antibiotic susceptibility test for *E. coli*

Antibiotic Disc	Disk Content (µg)	Antibiotic Susceptibility Pattern					
		Sensitive		Intermediate		Resistance	
		No.	%	No.	%	No.	%
Tetracycline (TE)	30	6	60	1	10	3	30
Cefotaxime (CTX)	30	7	70	-	-	3	30
Nitrofurantoin (NIT)	300	10	100	-	-	-	-
Ciprofloxacin (CIP)	5	3	30	-	-	7	70
Ampicillin (AMP)	10	6	60	-	-	4	40

Antibiotic susceptibility testing of Salmonella

The antibiotic susceptibility pattern of *Salmonella* isolates from eight different samples was determined and is shown in Table 4. Resistance pattern was observed in 37.5% of isolates against azithromycin and ceftriaxone, while 25% of isolates showed resistance pattern against cefixime and nalidixic acid. Similarly, 12.5% of isolates showed resistance patterns against ampicillin.

Table 4: Antibiotic susceptibility test for *Salmonella*

Antibiotic Disc	Disk Content (µg)	Antibiotic Susceptibility Pattern					
		Sensitive		Intermediate		Resistance	
		No.	%	No.	%	No.	%
Ampicillin (AMP)	10	4	50	3	37.5	1	12.5
Azithromycin (AZM)	15	5	62.5	-	-	3	37.5
Ceftazidime (CAZ)	30	5	62.5	-	-	3	37.5
Cefixime (CFM)	5	6	75	-	-	2	25
Nalidixic acid (NA)	30	3	37.5	3	37.5	2	25

Antibiotic susceptibility testing of Staphylococcus aureus

The antibiotic susceptibility pattern of *Staphylococcus aureus* isolates from six different samples was determined. All the isolates showed resistance against both ampicillin and cefoxitin. Similarly, 83.3% of isolates showed resistance against erythromycin. On the other hand, 50% of isolates and 33.33% of isolates showed resistance against vancomycin and ciprofloxacin, respectively. However, all the isolates were found to be sensitive to chloramphenicol. Out of the 10 samples collected, *Staphylococcus* was isolated from 6 samples and all the isolates showed MRSA upon screening.

Table 5: Antibiotic susceptibility test for *Staphylococcus aureus*

Antibiotic Disc	Disk Content (µg)	Antibiotic Susceptibility Pattern					
		Sensitive		Intermediate		Resistance	
		No.	%	No.	%	No.	%
Chloramphenicol (C)	30	6	100	-	-	-	-
Ampicillin (AMP)	10	-	-	-	-	6	100
Ciprofloxacin (CIP)	5	4	66.67	-	-	2	33.33
Erythromycin (E)	15	1	16.7	-	-	5	83.3
Vancomycin (VA)	30	3	50	-	-	3	50
Cefoxitin (CX)	30	-	-	-	-	6	100

Multidrug resistance patterns

Out of the 24 bacterial isolates identified, 7 (29.16%) exhibited multi-drug resistance in which one isolate was *E. coli* and the remaining 6 were *Staphylococcus* spp.

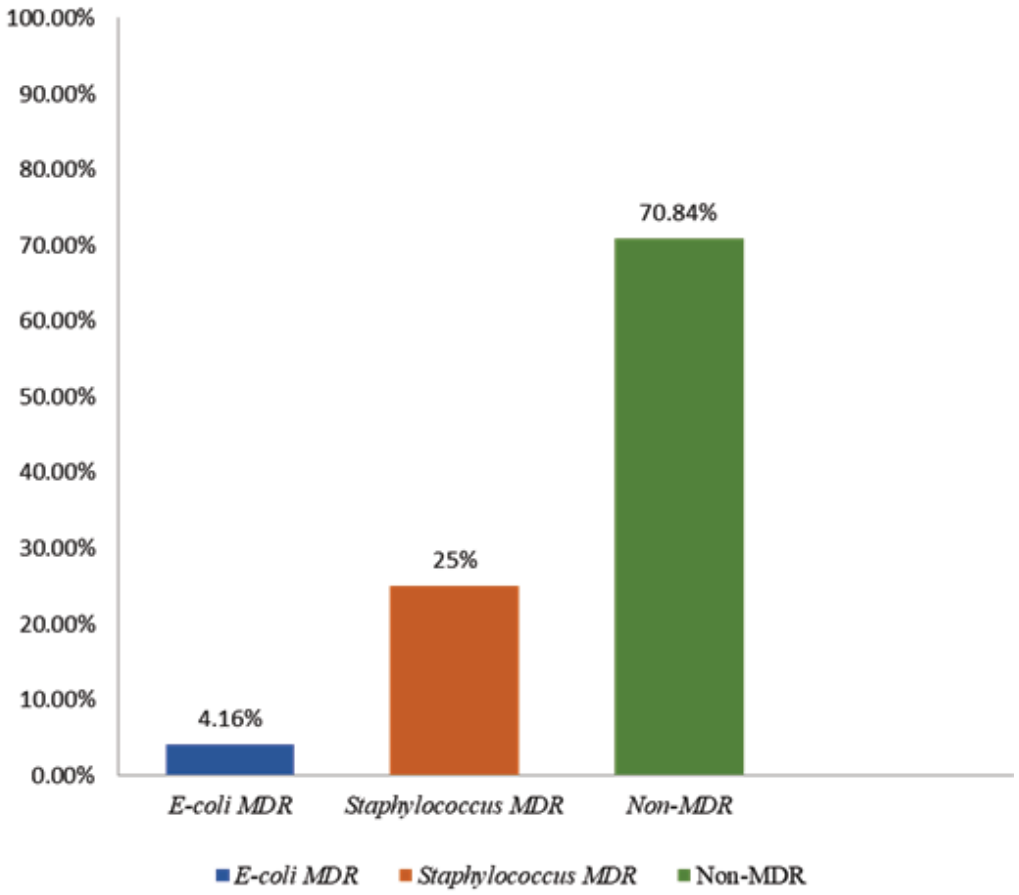

Figure 2: Multidrug resistance pattern

Discussion

The microbial quality of the chicken samples was assessed through total plate count (TPC) analysis. As shown in Table 1, the TPC values observed in raw chicken samples were notably higher than those previously reported by Kumari et al. (2019). Similarly, the TPC for frozen chicken meat ranged between 6.5 × 10^3 and 2.2 × 10^6 cfu/ml, also exceeding the microbial counts recorded by Kumari et al. (2019). Moreover, these findings surpass the generally accepted threshold for the total viable count in chicken meat, which is typically between 10^4 and 10^6 cfu/ml (Neupane & Kaphle, 2019). The elevated microbial load in frozen samples may indicate contamination in certain batches, posing a risk for spoilage over prolonged storage. The high contamination levels may result from poor hygienic handling and the use of contaminated water, underlining potential public health concerns.

The study revealed high levels of total coliforms (TCC) in all raw chicken meat samples and fecal coliforms (TFCC) in some, indicating microbial contamination due to poor hygiene during processing. The TCC values exceeded the acceptable range of 10^2–10^4 cfu/g, as mentioned by Neupane and Kaphle (2019), and were also higher than those reported by Kumari et al. (2019). Adhering to the guidelines given by the International Commission on Microbiological Specifications for Foods (ICMSF), the total coliform count (TCC) for raw meat surpasses the threshold of 2000 cfu/g, while the acceptable TCC for frozen meat should not exceed 100 cfu/g (Van Schothorst et al., 2009). Although not all raw samples contained TFCC, their presence in certain samples suggests potential fecal contamination and highlights inadequate sanitation practices (Harlia et al., 2017). In frozen meat, only one sample showed detectable coliforms, supporting previous findings that low temperatures can suppress coliform growth

(Nedwell, 1999). These results underscore the need for improved hygiene and cold-chain maintenance to ensure food safety.

The study revealed a high prevalence of *E. coli* (100%), *Salmonella* (80%), and *Staphylococcus* spp. (60%) in chicken meat samples, indicating significant microbial contamination. The presence of *E. coli* is consistent with the results of Abo-Almagd et al. (2023), which reported 98% of *E. coli* in the chicken carcasses examined. The highest contamination rates by *E. coli* may be due to unhygienic handling, improper slaughter practices, or the use of contaminated water during processing. The high prevalence of *Salmonella* (80%) is concerning given its role as a leading cause of foodborne illness. This may be attributed to differences in poultry farming practices, hygiene standards, and post-slaughter handling conditions. The presence of *Salmonella* in a majority of samples underlines the urgent need for interventions to control contamination throughout the poultry supply chain (Thapa et al., 2020). *Staphylococcus* spp. were found in 60% of samples, indicating potential contamination from human handlers, as these bacteria are common skin and nasal flora. The presence of *Staphylococcus* spp. may be due to poor hygiene during meat handling and packaging (Elmehrath et al., 2024). However, the absence of *Pseudomonas* species suggests the conditions were not favorable for its growth. These findings highlight the need for stringent hygiene and processing protocols in poultry production to ensure food safety and minimize health risks.

The study involved the evaluation of 10 *E. coli* isolates, and only one of them exhibited multidrug resistance. Antibiotic resistance patterns of *E. coli* isolates of this study align with studies done by Apun et al. (2008) and Rahman et al. (2008) which reported varying resistance levels to antibiotics such as ampicillin, tetracycline, and ciprofloxacin in Malaysia and Bangladesh, respectively. A study from Ecuador showed a high prevalence of multidrug resistance, with 98.3% of cefotaxime-resistant isolates also resistant to multiple other antibiotics. However, resistance to nitrofurantoin was low in that study, which aligns with the present findings, where no resistance to nitrofurantoin was detected, supporting its effectiveness against *E. coli* (Vinueza-Burgos et al., 2019).

The study involved the evaluation of 8 isolates, and none of them exhibited multidrug resistance. This signifies a relatively low prevalence of multidrug resistance among the tested isolates. A study conducted in Iran on retail chicken meat showed a high resistance to nalidixic acid (92.8%). However, in the study, a lower percentage of resistance to nalidixic acid was observed. Similarly, the research conducted by Bakhshi et al. (2017) reported lower resistance to cefixime. In the Iranian study, resistance to chloramphenicol (3.6%), amoxicillin-clavulanic acid (5.4%), and ampicillin (11.7%) was detected, but none of the isolates were resistant to ceftazidime, ceftriaxone, cefotaxime, ciprofloxacin, colistin, gentamicin, or imipenem. A global study on antibiotic resistance levels within the poultry production chain revealed that nalidixic acid and ampicillin faced the highest level of resistance (Castro-Vargas et al., 2020).

All six *Staphylococcus aureus* isolates showed a resistance pattern against ampicillin and cefoxitin. Similarly, 83.3% of isolates showed resistance against erythromycin. Similarly, a high resistance rate of 82.35% was observed against Erythromycin and 61.77% for chloramphenicol (Lika et al., 2021). On the other hand, 50% of isolates and 33.33% of isolates showed resistance against vancomycin and ciprofloxacin, respectively. However, all the isolates were found to be sensitive to chloramphenicol. However, it is noteworthy that the isolates showed a high susceptibility rate of 95.3% to ciprofloxacin, suggesting its effectiveness as a treatment option against the tested bacteria (Abunna et al., 2022). In addition the presence of MRSA in the poultry industry is a serious concern due to its risks to human health. MRSA strains that are resistant to antibiotics can produce enterotoxins that cause staphylococcal foodborne illnesses (Ribeiro et al., 2018). Recent research conducted by Abolghait et al. (2020) has highlighted an important discovery regarding MRSA isolates obtained from broiler chickens.

The presence of multidrug-resistant bacterial isolates is a significant concern, highlighting the growing issue of antibiotic resistance in foodborne pathogens. Resistance was observed in both *E. coli* and *Staphylococcus* isolates, indicating potential risks for treatment failure and compromised food safety.

This underscores the importance of monitoring and controlling antibiotic use in the poultry industry to prevent the spread of resistant strains and ensure effective treatment options remain available.

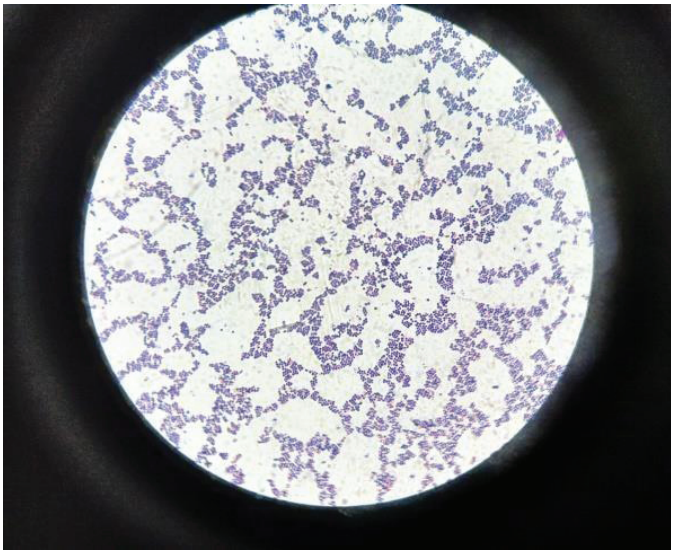

Figure 3: Microscopic view of Gram-positive cocci-shaped *S. aureus* from sample F1 under oil immersion

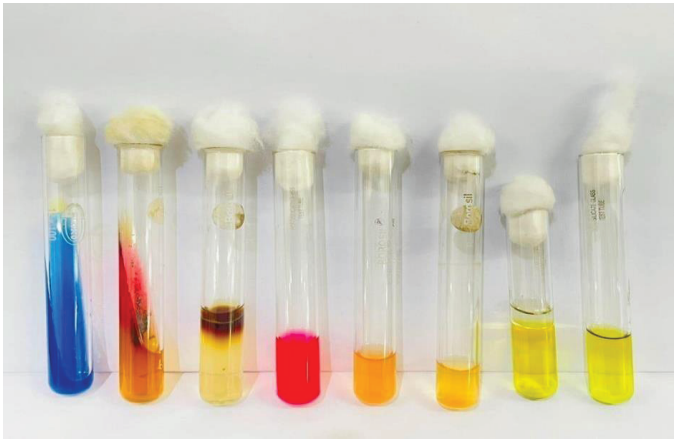

Figure 4: Biochemical test of *Salmonella* spp. from sample R2

From Left to right: Citrate (+), TSI (R/Y, G-, H2S+), SIM (S+I- M+), MR (+), VP (-), urease (-), O/F (Fermentative)

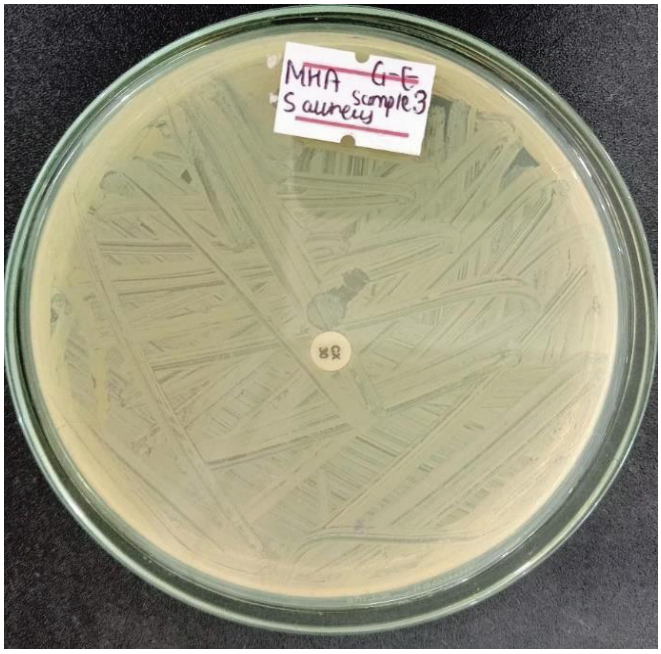

Figure 5: Methicillin-resistant *Staphylococcus aureus* with the antibiotic cefoxitin (30μg) on an MHA plate (sample F3)

Conclusion

This study provides conclusive evidence that raw chicken meat available in the market of Kathmandu valley exhibits significantly higher levels of microbial contamination compared to frozen chicken meat. The presence of such a high microbial load poses a considerable risk to public health, as these microbes can potentially harbor various pathogenic bacteria harmful to humans. The predominant bacteria isolated from the samples were *E. coli*, followed by *Salmonella* and *Staphylococcus*. This could be attributed to factors such as farm-to-flock sanitary practices, meat processing contamination, and environmental contact. Furthermore, the presence of multidrug resistance (MDR) and methicillin-resistant *Staphylococcus aureus* (MRSA) was detected in the *Staphylococcus* strains. Increasing antibiotic use has led to the emergence of resistance, reducing the effectiveness of drugs. Hence, antibiotic susceptibility tests emphasize the importance of limiting antibiotic usage.

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