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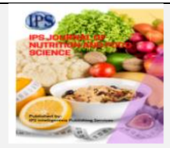

Molecular Analysis and *In Vitro* Pathogenicity Evaluation of Bacteria Isolated from Frozen Chicken

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Abstract
The consumption of frozen chicken has been associated with foodborne illnesses, posing significant public health concerns. Despite the importance of food safety, there is a dearth of information on the pathogenic potential of bacteria isolated from frozen chicken. This study aims to investigate the *in vitro* pathogenicity of bacteria from frozen chicken, addressing the gap in knowledge on the food safety implications of these microorganisms. Various strains of bacteria were isolated from frozen chicken samples collected from different locations in Awka Metropolis. The isolates were characterized based on their cultural, morphological, biochemical and molecular features. The pathogenic profiles of the isolates were evaluated using an *in vitro* approach. Statistical analysis was performed using the student "t" test and one-way analysis of variance (ANOVA). The bacterial isolates identified in the frozen chicken samples included *Staphylococcus aureus* strain C868, *Pseudomonas aeruginosa* strain 214, *Vibrio cholerae* strain 112Vc02 and *Salmonella enterica* subspecies *enterica* serovar Enteritidis. Significant pathological effects were observed in blood agar, and reaction with Congo red when the bacterial isolates were exposed using *in vitro* technique. This study demonstrates that different strains of bacteria isolated from frozen chicken samples exhibited distinct and varied pathological effects with *Vibrio cholerae* strain 112Vc02 showing the highest pathological features.

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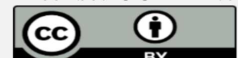

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1. Introduction

Freezing preserves food for extended periods by halting the growth and multiplication of microorganisms responsible for both food spoilage and foodborne illnesses. This preservation method also inhibits enzymatic activity within the food that would otherwise lead to deterioration. Most pathogens are unable to multiply at freezing temperatures, and many are inactivated due to the dysfunction of their enzymes, which are vital for sustaining cellular functions. In addition, freezing depletes the availability of water, a critical requirement for microbial growth, by converting it into solid ice crystals. This technique is particularly valuable in the preservation of meat, fish, and other animal protein-based products, as it extends shelf life while maintaining product quality in terms of dimensions, colour, flavour, and texture (Hafez *et al.*, 2020; Egurefa *et al.* 2020a; 2020b; Okolo *et al.*, 2025; Okpalaunegbu *et al.*, 2025; Obiefuna *et al.* 2025).

Foodborne pathogens (FPPs), including bacteria, fungi, and parasites, pose a substantial risk to public health when transmitted through contaminated food and water (Dwivedi and Jayku, 2011;Uba, 2019a; 2019b; 2019c; Uba *et al.*, 2019b; 2019c). These microorganisms are responsible for millions of foodborne disease cases each year. The World Health Organization (2020) estimates that approximately 600 million cases of foodborne illness occur globally on an annual basis, highlighting the critical nature of food safety. In recent years, the health burden associated with these pathogens has increased significantly, resulting in elevated morbidity and mortality across populations (Pennacchia *et al.*, 2011; Zhao *et al.*, 2014; Uba, 2019; Nwigwe *et al.* 2022, Nwigwe *et al.* 2023, Ifediegwu *et al.* 2023a; 2023b; Ifediegwu *et al.* 2024a, 2024b; 2024c; Orji and Oghonim, 2023; Nnaka *et al.*, 2024). Akeda (2015) emphasized that the risk is especially high in developing regions where food safety regulations are either inadequate or poorly enforced. Food contamination during processing may arise from physical (e.g., foreign materials), chemical, or biological sources, further compounding the problem (Anichebe *et al.*, 2019; Okoye *et al.* 2020a; 2020b; 2020c; Sadiku *et al.*, 2020; Uba *et al.* 2024; Mere *et al.* 2025; Enemchukwu *et al.* 2026a; 2026b).

Studies have shown that frozen foods harbour some pathogenic bacteria, which is a severe public health concern (Uba *et al*. 2016; Uba *et al.*, 2017; Uba *et al*. 2018a; 2018b; 2018c; 2019d; 2019e; Nkamigbo *et al*. 2020a; 2020b; Njoku *et al*. 2019a; 2019b; Uba *et al*., 2020a; 2020b; Alisa *et al*., 2020; Anukam *et al*., 2020a; 2020b; Umeh *et al.,* 2020; 2021; Uba e*t al.,* 2021a; 2021b; Dokubo *et al*., 2022a; 2022b; Anidu *et al*., 2023; Obiefoka *et al*., 2023; Ubajekwe *et al*., 2025; Uba *et al*., 2025) . Shamimuzzaman *et al*. (2022) reported the presence of *Escherichia coli* and *Salmonella* in ready-to-cook frozen foods, and they exhibited enough microbial loads and intoxication levels. Osaili *et al*. (2022) reported that there was an increase in the count of *Listeria monocytogenes* in frozen chicken samples after being stored at 4 ºC. Pathogens such as *Listeria monocytogenes*, *Salmonella spp.*, and *Escherichia coli* have been isolated from frozen meat, poultry, and seafood, indicating that improper freezing, thawing, or post-processing contamination may compromise food safety (Zhao *et al*., 2014).

Traditional microbiological methods can detect bacterial presence but often lack the precision needed to accurately identify species and assess their pathogenic potential. Molecular techniques provide more reliable identification and characterization of bacterial isolates, enabling the detection of virulence-related genes and improving understanding of their pathogenicity. Therefore, molecular analysis combined with *in vitro* pathogenicity evaluation is essential to determine the identity and virulence potential of bacteria associated with frozen chicken. This information is important for food safety monitoring, risk assessment, and the development of effective control strategies to reduce foodborne infections associated with poultry consumption.

2. Materials and Methods

2.1 Sample collection

A total of 56 random samples of frozen chicken meat cuts represented by wings, drumsticks, thigh and breast, weighing about 15 g for wing samples and 100-250 g for the other samples, were purchased from different supermarkets and cold rooms within Awka, Anambra State, Nigeria. Each sample was placed in a sterile plastic bag and transported immediately to the laboratory in a disinfected thermos flask. Frozen chicken was allowed to thaw at refrigeration temperatures before microbiological testing as described by several researchers (Dokubo and Uba, 2023; Uba and Obiefuna, 2023; Okafor *et al*., 2023; Ubani *et al*., 2024a; 2024b; 2025; Ekwenze *et al*., 2025; Ele *et al*., 2025; Uba and Okonkwo *et al*. 2025; Okwonkwo *et al.* 2026; Uba *et al.,* 2026a; 2026b; 2026c).

2.2 Sample preparation

The frozen chicken samples were sliced and 10 g sample macerated in 100 mL sterile peptone water, and further diluted to 1:10. One milliliter of the prepared sample was plated on Petri dishes (60 mm OD × 55 mm ID × 13mm high) containing Deoxycholate citrate agar medium (DCA/Biotech), Thiosulphate Citrate Bile Sucrose (TCBS) agar (BIOTECH), Cetrimide agar (BIOTECH) and Mannitol Salt Agar (MSA). All the plates in triplicates were incubated in inverted position at 37 ± 2˚C for 24 - 48 h. (Khalafallah *et al*., 2020; Alfred *et al*. 2023; 2025; Idu *et al*., 2026a; 2026b; Ibo *et al*. 2020; Ibe *et al*. 2023, Chukwura *et al*. 2025; Uba and Udaba *et al*. 2026; Dokubo and Uba, 2026).

2.3 Characterization and identification of bacterial isolates.

The bacterial isolates obtained were characterized and identified on the basis of morphological-and biochemical characteristics using the methods described by Cheesbrough, 2010. Further identification of the bacterial isolates was done using the 16SrRNA gene extraction, PCR and sequencing and blasting. The 16s rRNA amplified PCR products generated from universal primer(16s) which corresponds to the forward and reverse primers of 16SrRNA was used for the sequencing using ABI DNA sequencer (Applied Biosystem Inc) at International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria (Uba *et al*. 2020c; 2020d; 2020e; 2020f; 2020g; 2020h; Dokubo *et al*., 2024).

2.4 Screening the Bacterial Isolates for Pathogenic Potentialsusing *In vitro* Technique

The *in vitro* pathogenic potentials of the bacterial isolates was carried out by testing for the ability of the bacterial isolates to produce haemolysis on blood agar and took up Congo red dye as described in the study published by Zahid *et al*. (2016) and Ugwu *et al*. (2020).

2.4.1 Haemolysis on blood agar: Blood agar was prepared following the manufacturer's instruction, the medium was allowed to solidify and aseptically streaked with the test isolates. This was incubated in inverted position at 35±2 ℃ for 24 h. The presence of clear zones around the colonies was an indication of β haemolysis, partial zones of inhibition which was detected by greenish to gray zones was an indication of α-haemolysis whereas absence of clear zones is an indication for g-haemolysis as described by Zahid *et al*. (2016).

2.4.2 Reaction with Congo red dye: Nutrient agar was prepared following the manufacturer's instruction, this mixed with Congo red dye, and the medium was sterilized and allowed to solidified. The medium was aseptically streaked with the test isolates. This was incubated in inverted position at 35 ± 2℃ for 24 h. The presence of tiny red colonies with wrinkled surface was an indication of positive test whereas colonies with pink colour and smooth surface was an indication of negative result as described by Ugwu *et al*. (2020)

2.5 Statistical Analysis

The data were subjected to statistical analysis using IBM SPSS Version 23. Analysis of variance (ANOVA) was used to show the significant difference at $P < 0.05$ (Uba and Chukwura, 2016; Uba *et al.,* 2020h; Okafor *et al*. 2021a; 2021b; Ofunwa *et al*. 2024; Oghonim *et al.,* 2026; Okeke *et al*. 2025a; 2025b; Afulukwe *et al.,* 2025; 2026).

3. Results and Discussion

The cultural and morphological characteristics of the bacterial isolates are shown in Table 1. The isolates showed variations in their color, whereby isolate M appeared golden yellow in MSA, isolate P appeared bluish green in CA, isolate T appeared yellow in TCBS and isolate S appeared colorless with dark center in SSA. They exhibited similar features in their edges, surface, elevation and pigmentation. They are gram negative rods and motile except for isolate M which was gram positive cocci and non-motile. The characteristics of the bacterial isolates detected from the frozen chicken samples corroborated with findings of many other researchers (Ruban and Feuroze, 2011; Murad *et al*., 2014; Kunadu, 2018; Mahmoud *et al.*, 2020; Yar *et al*., 2020; Iheukwumere *et al*., 2012a; 2012b; Mundi *et al*., 2013; 2014; Okoye *et al*. 2014; Okoye *et al*. 2016a; 2016b; Uba *et al*., 2019a; Anameze *et al*., 2023; Ezeamama *et al*., 2025a; 2025b; Umezulora *et al.,* 2026).

The Biochemical characteristics of the isolates are shown in Table 2. The isolate were all catalase positive, isolate M, T and S were

oxidase negative except for isolate P which was positive, isolate M was coagulase positive while isolate P, T and S were coagulase negative. All isolates where indole negative and only isolate P was citrate negative. Isolate P, T and S were methyl red positive while isolate M was negative, isolate P, T and S were methyl red positive as they were able to utilize glucose in the medium to produce stable acids. Isolate M, T and S showed complete utilization of glucose whereas isolate P showed slight utilization, isolates M, Tand S showed complete utilization to maltose, xylose and mannitol except for isolate P which was not able to utilize the sugars. The isolates showed variations in their utilization of Sorbitol, Lactose and Inositol as shown in Table 2. The reaction of these isolates to the biochemical reactions agrees with the findings of Kunadu (2018) and Yar *et al*. (2020).

The nucleic acid extracted from the bacterial isolates revealed that they were deoxyribonucleic acid (DNA) as the ratio of 260 nm/280 nm ranged from 1.80 - 1.90 as shown in Table 3. Figure 1 showed the gel presentation of the gene amplicons while the molecular characteristics of the isolate revealed the identities of the isolates to be *Staphylococcus aureus, Pseudomonas aeruginosa* strain 214, *Vibrio cholerae* strain 112VCO2 and *Salmonella enterica sub species enterica* Serovar Enteritidis strain ECO20121750 as shown in Table 4. Several researchers detected these bacteria from their respective studies (Abd El-Tawab *et al*., 2015; Waturanji *et al*., 2015; Eriksson *et al.,* 2023). From the empirical review made, Abd El-Tawab *et al*. (2015) detected *Escherichia coli, Staphylococcus aureus* and *Salmonella* species, Waturanji *et al.* (2015) detected *Escherichia coli* and *Vibrio cholerae*, Shaltout *et al.* (2016) detected *Escherichia coli* and *Salmonella* species, Shaltout *et al.* (2018) detected *Staphylococcus aureus, Salmonella* species and *Escherichia coli;* all these findings agreed with the findings obtained from the present study.

The occurrences of the isolates revealed the *Pseudomonas aeruginosa* (PAP) recorded the highest occurrences with the percentage of 41.07% followed *Salmonella enterica* ser. Enteritidis (SSS) 28.57*%, Staphylococcus aureus* (SAM) and then *Vibrio cholerae* (VCT) as shown in Table 5. The survival of the bacterial isolates in the frozen environment supported the findings of many researchers (Mrityunjoy *et al.,* 2013; Odwar *et al.,* 2014; Waturanji *et al.,* 2015; Kunadu, 2018; Erikssun *et al.,* 2023).

The i*n vitro* pathogenicity of the isolates revealed that *Salmonella enterica* ser. Enteritidis (SSS) was the most Pathogenic isolate followed by *Pseudomonas aeruginosa* (PAP) as shown in Table 6. The significant pathogenic potential exhibited by the implicated bacterial isolates corroborated with the findings of many researchers (Elnawawi *et al* 2024; Mrityunjoy *et al.,* 2013; Sen and Carode, 2018; Zubar *et al.,* 2024). Waturanji *et al.* (2015) reported the pathogenic potential of *Vibrio cholerae* isolated from tested samples. The pathogenic potentials of these isolates could be attributed to their ability to release potent toxins or invasive enzymes that can lyse red blood cells or react with Congo red to produce benzidine. The production of this metabolite was the ability of these bacterial isolates to produce azoreductase that cleared the azoring of the Congo red, which corroborated the published report of Waturanji *et al.* (2015)

Table 1: Cultural and morphological characteristics of the bacterial isolates

Characteristics	M	P	T	S
Colour	Golden yellow in MSA	Bluish green in CA	Yellow in TCBS	Colourless with black center in SSA
Edge	Smooth	Smooth	Smooth	Smooth
Surface	Smooth	Smooth	Smooth	Smooth
Elevation	Raised	Raised	Raised	Raised
Pigmentation	Yes	Yes	Yes	Yes
Shape of Colony	Circular	Circular	Circular	Circular
Motility	Negative	Positive	Positive	Positive
Gram Reaction	Positive	Negative	Negative	Negative
Shape of Cells	Cocci	Rods	Rods and curved	Rods
Bacterium	*Staphylococcus*	*Pseudomonas*	*Vibrio*	*Salmonella*

Table 2: Biochemical Characteristics of the isolates

Characteristics	M	P	T	S
Catalase	+	+	+	+
Oxidase	-	+	-	-
Coagulase	+	-	-	-
Indole	-	-	-	-
Citrate	+	-	+	+
Methyl red	-	+	+	+
Voges proskauer	+	-	+/-	+/-
Glucose	+	+/-	+	+
Maltose	+	-	+	+
Xylose	+	-	+	+
Mannitol	+	-	+	+
Sorbitol	+/-	-	+/-	+/-
Lactose	+	-	+	-
Inositol	+/-	-	+/-	+
Bacterium	*S. aureus*	*P. aeruginosa*	*V. cholerae*	*S. enterica* serovar Enteritidis

Table 3: Nature of nucleic acid extracted from the isolates

Isolate	Conc (ng/ul)	280nm	260nm	260/280
SAM	101.40	1.6420	2.9884	1.82
PAP	98.80	1.6210	2.9826	1.84
VCT	121.20	1.7210	3.1839	1.85
SSS	109.60	1.6840	3.0817	1.83

Table 4: Molecular identities of the isolates

Isolates code	Max score	Total score	Query cover (%)	E value	Percentage identity	Accession number (%)	Description
SAM	2455	2455	100	0.0	100	CP127588.1	*Staphylococcus aureus* strain C868
PAP	1810	1810	100	0.0	100	CP115294.1	*Pseudomonas aeruginosa* strain 214
VCT	1552	1552	100	0.0	100	CP189269.1	*Vibrio cholerae* strain 112VC02 complete genome
SSS	2191	2191	100	0.0	100	CP007396.2	*Salmonella enterica* subspecies *enterica* serovar Enteritidis strain EC20121750

SAM PAP VCT SSS

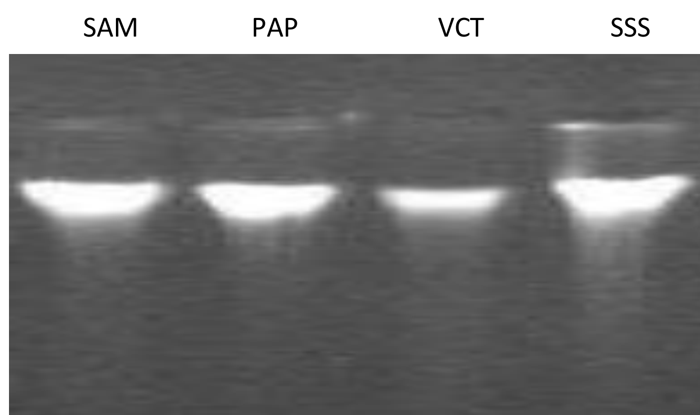

Figure 1: Gel presentation of the amplicons

Table 5: Occurrences of the isolates

Isolate	Occurrences	Percentage
Staphylococcus aureus M (SAM)	11	19.64
Pseudomonas aeruginosa P (PAP)	23	41.07
Vibrio cholerae T (VCT)	6	10.71
Salmonella enterica ser. Enteritidis S (SSS)	16	28.57
Total	56	100.00

Table 6: *In vitro* pathogenic potential of the isolates

Isolates	Hemolysis		
	β	α	γ
SAM (11)	9	2	0
PAP (23)	7	13	3
VCT (6)	6	0	0
SSS (16)	11	5	0
	Congo Red Reaction		
	Tiny Red with wrinkled surface	Pink/gray with smooth surface	
SAM (11)	11	0	
PAP (23)	21	2	
VCT (6)	6	0	
SSS (16)	16	0	

4. Conclusion

The presence of bacteria in frozen chicken highlights the potential risk of foodborne infections associated with poultry consumption. Although freezing helps preserve meat and reduce microbial growth, some pathogenic bacteria can survive and remain capable of causing disease. Molecular analysis provides accurate identification of bacterial isolates, while *in vitro* pathogenicity evaluation helps determine their virulence potential. Therefore, combining these approaches is essential for understanding the public health significance of bacteria present in frozen chicken. The findings of this study can contribute to improved food safety monitoring, better hygienic practices during poultry processing and storage, and the development of strategies aimed at reducing bacterial contamination and protecting consumer health.

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