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Research Article

Evaluation of Microbial Isolates from Frozen Chicken Products Sold in Port Harcourt Metropolis and Determination of their Antibiotic Susceptibility Patterns

Oritsetimeyin V Arueyingho*

Department of Pharmaceutical Microbiology and Biotechnology, University of Portharcourt, Nigeria

***Corresponding Author:** Oritsetimeyin V Arueyingho, Department of Pharmaceutical Microbiology and Biotechnology, University of Portharcourt, Nigeria.

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Abstract

The microbiological quality of different frozen chicken parts sold in Port Harcourt, Rivers State, was studied. Samples were collected from the open market. A total of two hundred chicken samples were collected and evaluated for microbial contamination. The samples were analysed in four different batches and their microbial load investigated, microorganisms that were isolated were properly characterised. The aims of the study are to isolate the microorganisms present in the frozen chicken samples, to characterize and identify the different microorganisms isolated from the samples and to determine the antibiotic susceptibility profiles of the isolates and their impact on public health. Pieces of frozen chicken parts were collected; 10g of the collected parts were shaken in universal tubes containing 10 ml of 0.1% peptone water with the aid of a mechanical shaker from which 10-fold serial dilutions were made. A 0.1 ml volume from each dilution was obtained in different batches and introduced into different growth media, which were incubated for 24hrs at a temperature of 37^0C and observed for growth of colonies. A total number of eleven microorganisms were isolated. The microorganisms isolated include: *Escherichia coli* (27.82%), *salmonella* sp (13.64%), *Shigella* sp (4.88%), *Staphylococcus aureus* (18.52%), *staphylococcus* sp (2.92%), *Bacillus subtilis* (17.83%), *Enterobacter* sp (4.15%), *Micrococcus* sp (1.61%), *Klebsiella* sp (1.84%), *Proteus* sp (3.07%) and *Citrobacter* sp (3.65%). The standard limit of all microorganisms contained in poultry falls within the range of 10^1- 10^2 CFU/g, however, the microbial load from the isolates ranged from 1.4-2.4 x 10^2 CFU/g which is outside the acceptable limits, hence, the samples analyzed were microbiologically unacceptable. Antibiotic susceptibility profile tests were subsequently carried out using four antibiotics: amoxicillin/clavulanate, gentamicin, ciprofloxacin and erythromycin. In conclusion, the microbial load in the analyzed samples were microbiologically unacceptable and certain microorganisms were observed to be resistant to the provided antibiotics; hence, proper sanitary practices, storage conditions and awareness programs should be implemented to encourage the provision of safe poultry products.

Keywords: Frozen Chicken; Standard Limit; Growth Media; Open Market

Introduction

Objectives of the study

To isolate the microorganisms present in the frozen chicken samples, to characterize and identify the different microorganisms isolated from the samples and to determine the antibiotic susceptibility profiles of the isolates and their impact on public health.

Rationale of the study

Due to the frequency of consumption of frozen chicken in the Nigerian populace, there is a possibility of increased risk of infection and change in the susceptibilities of the different microorganisms that could be isolated from these products and this could have a negative impact on the lives of the consumers. Hence, it is necessary to isolate, evaluate, characterize and identify the different microorganisms that are present in frozen chicken, so as to have a scientific proof of the risks associated with the consumption of frozen chicken, to advice the general public on health matters related to frozen chicken consumption. A research article by Vineetha., *et al*. explained that antibiotics are compounds synthesized naturally and artificially that have inhibitory action on other microorganisms. They are basically classified based on their mechanisms of action as: Cell Wall Inhibitors, Cell Membrane Inhibitors, Nucleic

acid synthesis inhibitors, Protein synthesis inhibitors and Metabolic Inhibitors. Based on these principles four (4) antibiotics were selected: Amoxicillin/clavulanate. erythromycin, gentamicin and ciprofloxacin.

Antimicrobial resistance

Antimicrobial resistance is the ability of certain microorganisms to resist the effects of an antimicrobial agent that was originally effective for the treatment of infections caused by them. Resistant microorganisms (including bacteria, fungi and viruses) are able to withstand attack by antimicrobial drugs, so that standard treatments become ineffective and infections persist, increasing the risk of spread to others.

Mechanisms of antimicrobial resistance

Mechanisms of gene transfer include transformation, transduction and conjugation. Transformation involves the uptake of DNA by transformable bacteria, the transfer of genes from one bacterium to another via bacteriophages occurs in transduction and the transfer of genes via a sexual pilus is the mechanism of conjugation.

Other mechanisms include interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis and disruption of bacterial membrane structure. Bacteria may be intrinsically resistant to more than one class of antimicrobial agents, or may acquire resistance by de novo mutation or via the acquisition of resistance genes from other organisms. Acquired resistance genes may enable a bacterium to produce enzymes that destroy the antibacterial drug [1].

Materials and Methods

The materials used include: culture media, reagents and commercial antibiotic discs.

Reagents

Peptone water, 1% tetramethyl p-phenylenediamine dihydrochloride, crystal violet, safranin red, iodine, hydrogen peroxide and kovac's reagent.

- **Culture media:** Macconkey agar, cetrimide agar, almonella-shigella agar. nutrient agar, nutrient broth, mannitol salt agar, mueller-hinton agar and sabouraud dextrose agar

Sample collection

A total of 200 samples of frozen chicken parts, were collected from the following locations:

- **Location A:** From the open markets of Rumuomoi, Obiwali market and a market at Nkpolu.
- **Location B**: From open markets in Rumuokoro and Choba
- **Location C**: Mile 1 and Mile 3 open markets
- **Location D:** Oil mill market.

Methods

Preparation of homogenate chicken samples

Various chicken parts were collected while wearing sterile gloves; they were wrapped in sterile foils which were placed in a cooler containing ice, hence trying to maintain the temperature at which they were collected before being transported to the laboratory. 10g of each of the various chicken parts were weighed and placed into 10 ml of 0.1% peptone water. This was afterwards shaken with the aid of a mechanical shaker for 15minutes. Serial dilutions of the slurry obtained were subsequently made in each sterile universal bottle containing 9 ml sterile 0.1% peptone water up to 10^{-10} dilution [2].

Culture methods

From each appropriate dilution 0.1ml was then inoculated on the following media as follows:

- **Nutrient Agar**: This was used for the enumeration of total bacteria isolates from the samples. The plates were incubated at 37˚C for 24 hours.
- **MacConkey Agar:** This was used for the enumeration of coliform bacteria in the samples. The plates were incubated at 37˚C for 24hours.
- **Sabouraud Dextrose Agar:** This was used for the enumeration of yeast isolates in the samples. The plates were incubated at 25˚C for 5-7 days (Shareef., *et al*. 2012).

Colonies from the incubated MacConkey agar and nutrient agar plates were picked and sub cultured on the salmonella-shigella agar, cetrimide and mannitol salt agar plates, these plates were then incubated at 37˚C for 24 hours. The colonies observed were noted and characterized. Once the microorganisms were isolated, they were preserved in agar slants which were kept in the incubator at 37˚C.

Morphological and biochemical tests

Several biochemical tests were carried out and they include Gram staining, indole test, catalase test, oxidase test and coagulase test.

Antibiotic susceptibility test

The Mueller-Hinton agar was prepared from a commercially available dehydrated base according to the manufacturer's instructions.

Immediately after autoclaving it was allowed to cool in a water bath, before being poured into plastic flat bottomed Petri dishes on

a level horizontal surface. The agar medium was then allowed to cool to room temperature and stored in a refrigerator.

At least three to five well isolated colonies of the same morphological type were selected from an agar plate culture. The growth was then transferred into a tube containing 4-5 ml of nutrient broth. The nutrient broth was then incubated until it achieves or exceeds the turbidity of the McFarland standard solution.

Inoculation of Test Plates: The swab was dipped into the inoculums suspension and then streaked on the dried surface of the Mueller-Hinton agar plate. The surface was streaked two more times rotating the plate approximately 60˚ each time to ensure an even distribution of the inoculums. Drug impregnated disks were then placed on the inoculate Mueller-Hinton agar plates after being exposed for about 3minutes. Each disc was pressed down to ensure complete contact with the agar surface. The plates were inverted and placed in an incubator set to 35˚C [3].

Preparation of stock solutions for antibiotic susceptibility testing using drugs available in the market

- **Amoxicillin/Clavulanate:** Amoksiklav Forte 312.5mg/5ml was used in the testing of the susceptibility of the microorganisms. This was diluted to obtain the working concentration. 5ml of the suspension said to contain 312.5mg of the drug was measured and transferred into a sterile bottle; the paper discs meant for the disc diffusion test were then dipped into the suspension, individually.
- **Ciprofloxacin:** The concentration of the infusion used was 2mg/ml this was diluted to obtain the working concentration, using a sterile pair of forceps each paper disc was dipped into the infusion.
- **Erythromycin**: Miral Erythromycin Suspension was used in the testing of the susceptibility of the microorganisms; this was diluted to obtain the working concentration. 5 ml of the suspension was measured and transferred into a sterile bottle; the paper discs meant for the disc diffusion test were then dipped into the suspension, individually.
- **Gentamicin:** The concentration of the drug in an ampoule is about 280mg/2ml, this was diluted to obtain the working concentration. The prepared paper disc was dipped into 2ml of the infusion individually and this was used for the disc diffusion test.

Preparation of dried filter paper discs

Whatman filter paper no. 1 was used to prepare discs approximately 6mm in diameter with the aid of a hole puncher, which were placed in a Petri dish and sterilised in the oven. A pair of forceps was then used to pick the discs which were individually dipped into the different antibiotics until they were properly soaked.

Drug impregnated disks were then placed on the inoculated Mueller-Hinton agar plates after being exposed for about 3minutes. Each disc was pressed down to ensure complete contact with the agar surface. The plates were inverted and placed in an incubator set to 35˚C [3].

Standardization of the antibiotic discs

The prepared antibiotics were tested for their efficacy using the Kirby-Bauer disc diffusion method and were checked if the diameter of the zone of inhibition was between the ranges for sensitivity of the organisms. Test organisms such as *Escherichia coli* and *Staphylococcus aureus* were used. They were sub cultured before the sensitivity testing. The inoculums were prepared from the cultures and were matched for turbidity with 0.5 Mcfarland solutions. The prepared antibiotic discs were placed on the inoculated agar plate along with the commercially available discs for comparison of the efficacy of the prepared discs. The plates were then incubated at 37˚C overnight. After incubation, the zones of inhibition were measured for each of the antibiotic discs and was seen if they were within the sensitivity range of the organisms.

Results and Discussion

The aerobic mesophilic count (total plate count) from each location was determined after sub culturing under optimum conditions. For each location, the average total number of microbial isolates were calculated per serial dilution and tabulated. The Total plate count for each location was more than the coliform count and the cell count reduced as the serial dilution increased. (Table 1)

The morphology of the colonies observed on the plate were noted and described, some of them were mucoid, some were coloured and some had characteristic features. The cell shapes when observed underneath the microscope were either rods or cocci. Several biochemical tests were carried out and this aided in the identification of the microbial isolates; all these make up the characterization process.(Table 2)

The percentage of each microorganism in the entire population was calculated, based on the above results which show the average cell count of each microorganism per location. The percentages are as follows: *Escherichia coli* (27.82%), *salmonella* sp (13.64%), *shigella* sp (4.88%), *Staphylococcus aureus* (18.52%), *staphylococcus* sp (2.92%), *Bacillus subtilis* (17.83%), *enterobacter* sp (4.15%), *micrococcus* sp (1.61%), *klebsiella* sp (1.84%), *proteus* sp (3.07%) and *citrobacter* sp (3.65%). (Table 3)

Colony morphology	Cell character	Gram staining	Indole test	Catalase test	Oxidase test	Probable identity	Locations
Mucoid	Short rod	-	+	+	-	*Escherichia coli*	A, B, C
Large white mucoids	Rods arranged in chains.	+	-	+	+	*Bacillus subtilis*	A, B, C And D
Yellow, small and irregular	Cocci	+	-	+	-	*Staphylococcus aureus*	A, B, C And D
Small white mucoid	Rods	-	+	+	-	*proteus sp*	A, B
Large white and mucoid on nutrient agar, black centres observed in selective media	Rods	-	-	+	-	*salmonella sp*	A, B, C And D
Pale almost translucent colonies	Rods	-	-	+	-	*shigella sp*	B, C, D
Small and raised	Rods	-	-	+	-	*klebsiella sp*	A, B
Pale pink colonies	Rod	-	-	+	-	*enterobacter*	A, C and D
Light red small colonies	Cocci	+	-	+	-	*micrococcus sp*	A, C
Moist, low, smooth and translucent	Rod	-	-	+	-	*citrobacter sp*	B, C and D
Small white colonies	Cocci	+	-	+	-	*staphylococcus sp*	A,C and D

Table 1: Results on the morphological and biochemical tests from the isolates of the frozen chicken in all locations.

Microorganism	Location A (Average Cell count)	Location B (Average Cell count)	Location C (Average Cell count)	Location D (Average Cell count)	Total Average Cell Count in all Locations per Microorganism
Escherichia coli	150	180	200	195	725
Salmonella sp	80	60	95	120	355
Shigella sp	-	20	35	72	127
Staphylococcus aureus	102	98	122	160	482
Staphylococcus sp	28	-	36	12	76
Bacillus subtilis	68	44	202	150	464
Enterobacter sp	22	-	36	50	108
Micrococcus sp	14	-	28	-	42
Klebsiella sp	12	36	-	-	48
Proteus sp	20	60	-	-	80
Citrobacter sp	-	-	50	45	95
					Total = 2602

Table 2: Average cell count of each Microorganism per Location.

Antibiotic susceptibility test results				
Organism	***Escherichia coli***		***Staphylococcus aureus***	
Antibiotic	**P (in mm)**	**C (in mm)**	**P (in mm)**	**C (in mm)**
Amoxicillin/ Clavulanate	20	18	18	18
Erythromycin	-	-	22	20
Gentamicin	24	22	25	30
Ciprofloxacin	34	30	26	24

Table 3: Zones of Inhibition values for the used antibiotics.

Where P= Prepared antibiotics; C= Commercial antibiotic

The efficacy of the prepared antibiotic discs was studied by comparing them with the commercially obtained antibiotic discs using standard strains of different bacteria. They were compared for their zones of inhibition and the above results were obtained. (Table 4 and 5)

The susceptibility and resistance patterns of the microorganisms to the antibiotics were noted after the diffusion test was carried out. From the table it could be observed that different antibiotics had different actions on the microorganisms E.g. *Staphylococcus aureus* was completely resistant to all antibiotics and *Klebsiella* sp was susceptible to all antibiotics used.

Microorganism	Amoxicillin/Clavulanate	Erythromycin	Gentamicin	Ciprofloxacin
Bacillus subtilis	S	S	R	S
Staphylococcus aureus	R	R	R	R
Staphylococcus sp	S	S	R	S
Citrobacter sp	R	S	S	S
Enterobacter sp	R	R	S	S
Escherichia coli	R	R	S	S
Micrococcus sp	S	R	S	R
Salmonella sp	S	R	S	S
Shigella sp	S	S	S	S
Proteus sp	R	S	R	S
Klebsiella sp	R	S	S	S

Table 4: Antibiotic susceptibility testing results obtained using Commercial Pure Antibiotic discs.

Microorganism	Amoxicillin/Clavulanate	Erythromycin	Gentamicin	Ciprofloxacin
Bacillus subtilis	S	S	R	S
Staphylococcus aureus	R	R	R	R
Staphylococcus sp	R	S	R	S
Citrobacter sp	R	S	R	S
Enterobacter sp	S	R	R	S
Escherichia coli	R	R	S	S
Micrococcus sp	S	R	S	R
Salmonella sp	S	R	S	S
Shigella sp	S	S	I	S
Proteus sp	R	S	R	S
Klebsiella sp	S	S	S	S

Table 5: Antibiotic susceptibility testing results obtained using drug products obtained from the market.

Where S= Susceptible; R= Resistant; I= Intermediate

A total of 11 bacteria species from the various frozen chicken parts examined were isolated of which 4 (four) were gram positive and 7 (seven) gram negative. These organisms are also sources of diarrhoea and/or gastro intestinal disturbance to both adults and children when consumed. This is in agreement with the findings of others [4,5] concerning frozen chicken stored under different conditions [2]. It was noted that Escherichia coli was present in the samples collected from each different location, while location C had the highest amount of *Escherichia coli* isolated, location A had the least amount, with an average of about 150 Coliform cells counted on the Macconkey agar plate.

Citrobacter sp was not isolated from the frozen chicken samples analyzed from the first two locations. It could also be observed that other species of *staphylococcus, Enterobacter, micrococcus, klebsiella, proteus* and *citrobacter* were isolated but in minute amounts, compared to *Escherichia coli, salmonella, Staphylococcus aureus* and *Bacillus subtilis*. Hence, the microorganisms, *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, proteus* sp, *salmonella* sp, *klebsiella* sp, *enterobacter* sp, *micrococcus* sp and other species of *staphylococcus* were found in all locations. The prevalence of the microorganisms isolated from each location where noted [6-40].

Conclusion

In conclusion, the resistance profile of the isolated microorganisms which were not only quantified but characterized and identified was obtained, and it was observed that the pure antibiotic discs were more potent than the commercially prepared antibiotics.

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