Nig. J. Pure & Appl. Sci. Vol. 33 (Issue 2, 2020)
eISSN 2756-4045

www.njpas.com.ng

doi: http://dx.doi.org/10.48198/NJPAS/20.A03

Study of antibiotics resistance in bacteria isolated from retailed eggshell in three major markets in Minna, Nigeria

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ABSTRACT

Food borne disease associated with inappropriately treated or untreated eggs is a major public health problem affecting developing and developed countries. This study isolated, identified and determined the Antibiogram of bacteria isolates from eggshell of eggs obtained from three major markets in Minna, Nigeria. A total of ten (10) duplicate egg samples were purchased from egg retailers. The egg samples were analyzed for the enumeration and isolation of bacteria. The isolated bacteria were identified using biochemical methods. Antibiotic susceptibility test to prescribed commercially available antibiotic discs was also investigated. The total viable bacteria count (TVBC) ranged from 1.38×10^4 - 2.52×10^4 cfu/mL while the total coliform count (TCC) ranged from 8.5×10^3 - 2.02×10^4 cfu/mL. *Escherichia coli* (9.1%), *Streptococcus pyogenes* (4.5%), *Enterococcus* sp. (4.5%), *Shigella* sp. (13.6%), *Salmonella* sp. (13.6%), *Staphylococcus aureus* (36.4%), *Clostridium* sp. (9.1%) and *Neisseria* sp. (9.1%) were isolated and identified from eggshells. Gram positive bacteria showed highest sensitivity to gentamycin (100%) however, resist cloxacillin, ceftadizime and erythromycin (100%). Similarly, there was no ciprofloxacin resistant Gram-negative bacteria though *E. coli, Salmonella* sp. and *Shigella* sp. isolated from eggshells were resistant to augmentin and amoxicillin. Further analysis of result revealed that all the isolated bacteria from eggshells were multidrug resistant except *Neisseria* sp. with multidrug resistant index greater than 0.2. The fact that these antibiotic resistant bacteria can be transferred to humans is of public health concern. Therefore, stringent use of public health regulations for cleaning eggs before retailing is advocated

Keywords: Antibiotic resistance, egg retailers, eggshells, cloxacillin

Introduction

Eggs constitute an important part of the human diet due to its high-quality of proteins, which makes them nutritious (Alba *et al*., 2015). The interior of the egg is protected by the shell, making it unsusceptible to microbial contamination. However, the shell is always soiled with blood, manure, feathers and nest materials (Rehana *et al*., 2017). Such substances are most likely contaminated with bacteria, especially through the fecal materials.

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It was observed that about 5-12% of eggshells are internally contaminated within few hours after they are laid (Rehana *et al*., 2017). The organisms of which some are pathogenic, are deposited on the shell from the intestinal tract of the hen and also from the environment making the egg shells a reservoir of pathogenic bacteria (Jannatul *et al*., 2016). In the absence or poor implementation of public health regulations for cleaning eggs before retailing as is the case in most developing countries like Nigeria, handlers and consumers are at risk of contracting potentially pathogenic bacteria contaminant from contaminated eggshell surfaces.

Poultry birds are often administered antibiotics at sub-therapeutic doses to promote their growth and keep them healthy, nevertheless, these antibiotics can cause the microorganism in the gut of these birds to develop resistance by mutating or picking up resistance genes (Oviasogie *et al.*, 2016). Recent studies have demonstrated the isolation of resistome from poultry farms. However, there is paucity of data on the microbiological quality of eggs sold in markets in Nigeria. This study was designed to isolate, identify and determine the antibiogram profile of bacteria isolates from eggshells in three different markets in Minna, Nigeria.

Materials and Methods

Study Area

Minna, the capital of Niger state has about 286, 838 individuals as at 2006 (Ademola *et al.,* 2018). Minna lies between the Longitude 3º30º E and 7º20ºN, and Latitude 8º20º N and 11º30º N. The total land mass of Minna is approximately 74,344km^2 in width which covers a total of 8% of the land area in Nigeria (Ademola et al., 2018). Minna is one of the developed urban centres in the North-Central geopolitical zone of Nigeria. In Minna, the residential densities are homogeneous comprising of high, medium and low-income earners.

Sample collection

Three duplicated eggs samples were randomly purchased from Tunga, Bosso and Kure markets in Minna, Nigeria. One extra sample was purchased from Kure market being the largest of the three-market area sampled. The egg samples were packed in a sterile sample container and moved to the Laboratory of the Department of Microbiology, Federal University of Technology Minna, Nigeria for microbiological analysis.

Preparation of inoculum

For each eggshell, one sterile swab stick was made wet in sterile peptone water. Shell swab was taken from the entire surface of the egg and immediately inoculated in a test tube containing 9 mL of peptone broth for sample enrichment (Abdullah, 2010). The inoculated peptone broth was incubated at 37ºC for 24 hours.

Enumeration of total counts

Zero point one mililitre (0.1 mL) of the inoculum was plated on MacConkey agar and Nutrient agar for total coliform bacteria count and total viable bacteria count. The plates were incubated for 24 hrs at 37°C. All samples were plated on duplicate plates. The colonies that appeared at the end of incubation period were counted using digital illuminated colony counter and result was expressed in colony forming units per mL (cfu/mL) of the samples (Fardows *et al*., 2016).

Isolation of bacterial contaminants

A loopful of inoculum was streaked onto blood agar medium, Salmonella Shigella Agar (SSA) and Mannitol Salt Agar (MSA). The inoculated plates were incubated at 37ºC for 24 hours. The plates were observed for visible bacteria colonies and the discrete bacteria colonies were sub-cultured on fresh sterile Nutrient agar plates to obtain pure isolates. Pure isolates were subcultured on sterile agar slants and stored at 4ºC for further use.

Identification of organisms

The isolated bacteria were identified by their colony morphology, Gram staining characteristics and biochemical tests: catalase, coagulase and oxidase, indole, hydrogen sulphide test, methyl red, citrate and urease test as described by Cowan and Steel (2002); Cheesebrough (2006); and Loongyai *et al*. (2010).

Antibiotic susceptibility test

Discs of antibiotics (RapidLab: LOT SK03/P) commonly used for the treatment of bacterial infection (Gram negative and positive) infection were used. Individual colonies were suspended in normal saline to 0.5 McFarland standards using sterile swabs. The suspensions were inoculated on Muller Hinton agar (MHA) using a sterile swab stick. The antibiotic discs were placed at 25 mm spacing apart (4 discs per agar plate) on the inoculated MHA plates. Sterile 6 mm Whatman filter paper No. 3 (Germany), impregnated with sterile distilled water was used as control.

The diameter of the zones of inhibition around the antibiotic discs, the organisms were classified as susceptible, intermediate, or resistant to a specific antibiotic (CLSI, 2018). A multidrug resistant (MDR) bacterial pathogen was defined by resistance to ≥1 agent in ≥3 antimicrobial classes (CLSI, 2018). Multiple Antibiotic Resistance Index (MARI) (in respect to a single isolate was defined as a/b, where "a" represents the number of antibiotics to which the isolate was resistant and "b" represents the number of antibiotics to which the isolate was exposed), was also calculated (Liberto *et al*., 2009). The various classes of antibiotics, including cloxacillin ampicillin, amoxycillin/clavu la-nate, ofloxacin, ciprofloxacin, gentamycin, nitrofurantoin, erythromycin, ceftazidime, cefuroxime, and ceftriaxone were used in this study for the antibiotic susceptibility test, as shown in Table 1. The CLSI chart was used to determine the susceptibility pattern of the organism to the different antibiotics used.

Data Analysis

Data obtained from this study were analyzed using statistical package for social sciences (SPSS) version 24. Cross tabulation performed to determine the relationship between the rate of the resistant, intermediate and susceptible pattern of the isolated bacteria to the antibiotics. Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were used to determine the significant differences between the bacteria load of eggshells from the three major markets. p >0.05 was considered statistically significant.

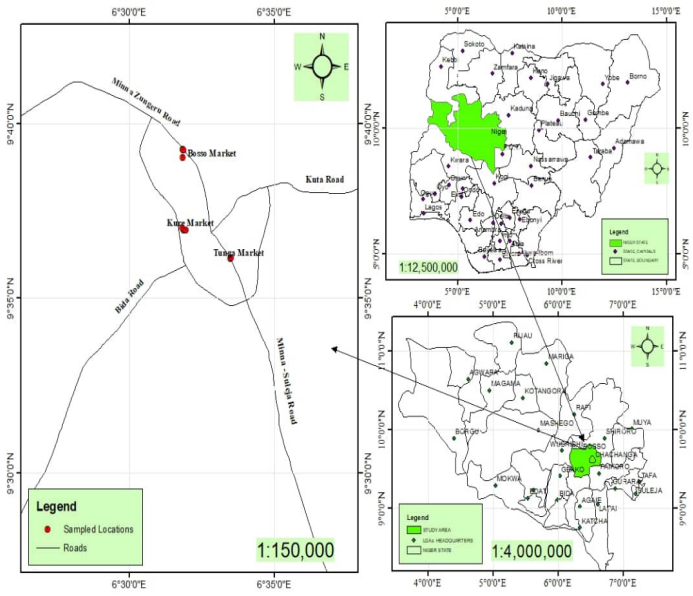

Figure 1: Study area and distribution of markets included in the study

Table 1: Classes of antibiotics used.

Classes of antibiotics	Antibiotics
Penicillin	cloxicillin (CXC), ampicillin (PN) and amoxycillin/clavulanate (AUG)
Quinolones	ofloxacin (OFX) and ciprofloxacin (CPR)
Aminoglycoside	gentamycin (GEN)
Nitrofurans	nitrofurantoin (NIT)
Macrolides	erythromycin (ERY)
Cephalosporins	ceftazidime (CAZ), cefuroxime (CRX) and ceftriaxone (CTR)

Results

Microbial load of retailed eggshells in Minna, Nigeria

The total viable bacteria count (TVBC) ranges from 1.38×10^4 cfu/mL in sample 3 to 2.52×10^4 cfu/mL in samples 2 and 10. Whereas, the total coliform count (TCC) ranges from 8.5×10^3 cfu/mL in sample 6 to 2.02×10^4 cfu/mL in samples 1 and 3 (Table 1).

Frequency of occurrence of bacterial isolates from retailed eggshells from three major markets in Minna

The pure bacteria colonies from eggshells of retailed eggs were identified as *E. coli* (9.1%), *S. pyogenes (*4.5%), *Enterococcus* sp. (4.5%), *Shigella* sp. (13.6%), *Salmonella* sp. (13.6%), *S. aureus* (36.4%), *Clostridium* sp. (9.1%) and *Neisseria* sp. (9.1%) (Table 2 and Figure 1).

Antibiogram of bacterial isolates from retailed eggshells from three major markets in Minna

S. pyogenes, S. aureus, Enterococcus sp. and *Clostridium* sp. from retailed eggshells resist the antibacterial effect of erythromycin and coxacillin. However, they were susceptible to gentamycin. Majority (>50) of the isolated *S. pyogenes, S. aureus* and *Clostridium* sp. resisted ciprofloxacin whereas, the 8 *Enterococcus* sp. isolated from retailed eggshells showed intermediate resistance to ciprofloxacin. *Clostridium* sp., *S. pyogenes* and *Enterococcus* sp. were resistant to ceftadixime.

Except for *S. aureus* (25%) and (37.5%) that demonstrated varying resistance, *S. pyogenes, S. aureus* and *Clostridium* sp. were susceptible to ofloxacin and cefuroxime (Table 3). Similarly, *S. aureus* was resistant to gentamycin.

E. coli, Salmonella sp. and *Shigella* sp. isolated were resistant to augmentin and amoxicillin but susceptible to gentamycin, ofloxacin and ciprofloxacin. *Neisseria* sp. (50%) isolated was resistant to ceftadizime, augmentin, cefuroxime and ampicillin though, susceptible to ofloxacin, gentamycin, ciprofloxacin and nitrofurantoin (Table 4).

Except for *Neisseria* sp., all the bacterial isolates from this study may be exhibiting multidrug resistance with MARI greater than 0.2 (Table 5).

Table 1: Mean value of colony forming units per milliliter from retailed eggshells from three major markets in Minna.

Sample Code	TVBC (cfu/mL) $\times 10^4$	TCC (cfu/mL) $\times 10^4$
1	$1.52^{d}\pm0.03$	$2.02^{a}\pm0.02$
2	$2.52^{a}\pm0.03$	$2.01^{a}\pm0.02$
3	$1.38^{e}\pm0.03$	$2.02^{a}\pm0.03$
4	$1.95^{b}\pm0.02$	$1.62^{ab}\pm0.02$
5	$1.52^{d}\pm0.02$	$0.925^{b}\pm0.03$
6	$2.01^{b}\pm0.01$	$0.85^{b}\pm0.07$
7	$2.01^{b}\pm0.03$	$1.51^{ab}\pm0.02$
8	$1.72^{c}\pm0.02$	$1.42^{ab}\pm0.02$
9	$1.72^{c}\pm0.02$	$1.02^{b}\pm0.03$
10	$2.52^{a}\pm0.02$	$1.51^{ab}\pm0.01$

Values are $\bar{x}\pm$SEM of duplicate determinations. Means with dissimilar letter (s) differ significantly according to the Duncan Multiple Range Test (DMRT) **$p \leq 0.05$.** Key: TVBC = Total Viable Bacteria Count and TCC = (Total Coliform Count)

Table 2: Morphological and biochemical characteristics of bacterial isolates from retailed eggshells from three major markets in Minna

Sc	GR	Sh	Ct	Cit	H_2S	Mot	Nit	MR	VP	Ure	Oxi	Ind	Glu	Coa	Lac	Man	Inference
1	+	C	+	+	-	-	+	+	+	+	-	-	+	+	+	+	*Staphylococcus aureus*
2	-	C	+	-	+	+	+	+	-	-	-	-	+	NA	-	+	*Salmonella* sp.
3	-	R	+	-	-	-	+	+	-	-	-	+	NA	-	-	+	*Shigella* sp.
4	+	C	-	-	-	-	+	-	+	-	-	-	+	-	+	+	*Enterococcus* sp.
5	+	C	-	NA	NA	-	NA	NA	-	-	-	NA	+	NA	+	-	*Streptococcus pyogenes*
6	-	R	+	-	-	+	+	+	-	-	-	+	+	NA	+	+	*Escherichia coli*
7	-	R	-	NA	-	+	-	NA	NA	-	+	N	+	NA	-	NA	*Neisseria* sp.
8	+	R	-	NA	+	+	-	NA	-	-	-	-	+	NA	-	-	*Clostridium* sp.

Key: Sc (Isolate code), GR (Gram Reaction), Sh (Shape), Ct (catalase), Cit (Citrate), H_2S (Hydrogen Sulphide), Mot (Motility), Nit (Nitrate), MR (Methyl Red), VP (Vorges Proskauer), Ure (Urease), Oxi (Oxidase), Ind (Indole), Glu (Glucose), Coa (Coagulase), Lac (Lactose) and Man (Manitol), C (Cocci), R (Rod), + (Positive), - (Negative) , NA (Not applicable), Sc (Sample code)

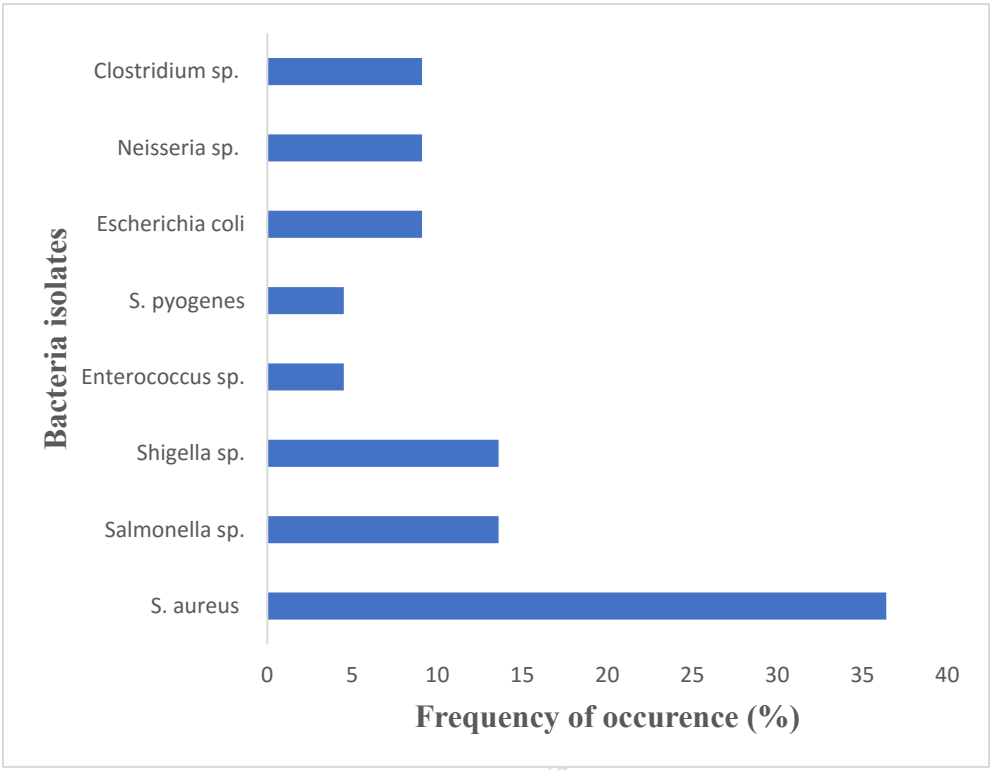

Figure 1: Frequency of occurrence of bacteria isolated from retailed eggshells from three major markets in Minna

Table 3: Antibiogram profile of Gram-positive bacteria isolated from retailed eggshells from three major markets in Minna

Bacteria isolate	Number of isolates	Pattern	CPR (%)	CAZ (%)	OFL (%)	ERY (%)	CRX(%)	AUG (%)	GEN (%)	CXC (%)
		I	1(50)	0(0)	0(0)	0(0)	0(0)	2(100)	0(0)	0(0)
Clostridium sp.	2	R	1(50)	2(100)	0(0)	2(100)	0(0)	0(0)	0(0)	2(100)
		S	0(0)	0(0)	2(100)	0(0)	2(100)	0(0)	2(100)	0(0)
		I	1(100)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)
Enterococcus sp.	1	R	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)	0(0)	1(100)
		S	0(0)	0(0)	0(0)	0(0)	1(100)	1(100)	1(100)	0(0)
		I	2(25)	0(0)	1(12.5)	0(0)	1(12.5)	0(0)	0(0)	0(0)
S. aureus	8	R	4(50)	7(87.5)	1(12.5)	8(100)	2(25)	6(75)	0(0)	8(100)
		S	2(25)	1(12.5)	6(75)	0(0)	5(62.5)	2(25)	8(100)	0(0)
		I	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. pyogenes	1	R	1(100)	1(100)	0(0)	1(100)	0(0)	1(100)	0(0)	1(100)
		S	0(0)	0(0)	1(100)	0(0)	1(100)	0(0)	1(100)	0(0)
P-value			0.677	0.909	0.398	---	0.920	0.017	---	---

Key: I (Intermidiate), R (Resistant), S (Susceptible)

Table 4.4: Antibiogram profile of Gram-negative bacteria isolated from eggshells

Bacteria isolate	Number of isolates	Pattern	CRX (%)	NIT (%)	OFL (%)	CAZ (%)	AUG (%)	GEN (%)	AMP (%)	CPR (%)
		I	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. coli	2	R	2(100)	0(0)	0(0)	2(100)	2(100)	0(0)	2(100)	0(0)
		S	0(0)	2(100)	2(100)	0(0)	0(0)	2(100)	0(0)	2(100)
		I	1(50)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Neisseria sp.	2	R	0(0)	0(0)	0(0)	1(50)	1(50)	0(0)	1(50)	0(0)
		S	1(50)	2(100)	2(100)	1(50)	1(50)	2(100)	1(50)	2(100)
		I	1(33.3)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Salmonella sp.	3	R	1(33.3)	0(0)	0(0)	0(0)	3(100)	0(0)	3(100)	0(0)
		S	1(33.3)	3(100)	3(100)	3(100)	0(0)	3(100)	0(0)	3(100)
		I	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Shigella sp.	3	R	3(100)	1(33.3)	0(0)	0(0)	3(100)	0(0)	3(100)	0(0)
		S	0(0)	2(66.7)	3(100)	3(100)	0(0)	3(100)	0(0)	3(100)
p. value			0.301	0.459	---	0.055	0.217	---	0.217	---

Key: I (Intermidiate), R (Resistant), S (Susceptible)

Table 4: Multidrug resistance and MAR index of the isolated bacteria

Isolates	MDR resistance pattern	MAR index
Clostridium sp.	CTR/CAZ/ERY/CXC	0.4
Enterococcus sp.	CAZ/ERY/CXC	0.4
S. aureus	CAZ/CRY/CXC	0.4
S. pyogenes	CTR/CAZ/ERY/AUG/CXC	0.6
E. coli	CRX/CAZ/AUG/AMP	0.5
Neisseria sp.	CAZ/AUG/AMP	0.4
Salmonella sp.	AUG/AMP	0.3
Shigella sp.	CRX/AUG/AMP	0.4

Discussion

The result obtained from this study revealed that shell of eggs available for consumers in the three major markets in Minna Metropolis were heavily contaminated (Table 1). The high microbial counts of egg shell samples indicate poor hygienic conditions under which the eggs were laid, handled and stored. More so, the lack of adequate refrigeration during the market storing in the developing countries, especially in Nigeria, may have contributed greatly to the level of different bacterial contamination on egg shell. Previous reports by Fardows *et al*. (2016) and Oviasogie *et al*. (2016) opined that microbial contamination is common among retailed eggs sold in markets in Nigeria. Therefore, the result of this study is in tandem with previous reports.

E. coli, *S. pyogenes, Enterococcus* sp., *Shigella* sp., *Salmonella* sp., *S, aureus, Clostridium* sp. and *Neisseria* sp. were isolated and identified in this study (Table 2). The fact that the isolated bacteria are potentially food borne pathogens reflect negatively on public health in Minna (Momani *et al*., 2018). *S. aureus* (36.4%) dominated the flora on retailed eggshell in the study areas. This might be attributed to their tolerance to dry condition (De Reu *et al*., 2007) or suggest egg contamination due to poor hygienic practices. This result is in concordance with the one obtained by Fardows *et al*. (2016); Ibrahim *et al.* (2014); Siriporn *et al*. (2015), where *S. aureus* had the highest frequency of occurrence compared to other bacteria isolated from the shell of eggs.

The presence of primary fecal indicator (*E. coli*) and other coliforms such as *Shigella* sp. (13.6%) and *Salmonella* sp. (13.6%) were recovered from the sampled eggshells. The shell is the most exposed part of the egg thus, *Shigella* sp. and *Salmonella* sp., which are enteric commensals, may have been transferred to the egg during the laying process or by trans-shell contamination with faecal material of animal and/or human origin (Pattison *et al*., 2008; Jambalang *et al*., 2017).

Furthermore, the occurrence of these food borne bacterial pathogens on eggshells retailed from major markets in Minna pose serious threat to public health. This result disagrees with the reports from similar studies conducted by Ibrahim *et al.* (2014); Chaemsanit *et al*. (2015); Mansour *et al.* (2015); Al Momani *et al*. (2017); and Parveen *et al.* (2017), where *Shigella* sp. was not isolated. The reason for this variation could be difference in the level of hygienic practices by egg handlers or diseased state of the birds that laid the eggs. Since, the egg is most often contaminated by faeces, soil, litter or equipment after laying (Svobodová and Tůmová, 2014).

The low frequency (9.1%) of *Neisseria* sp. recorded in this study is slightly higher than 5% reported by Chaemsanit *et al*. (2015). However, *Neisseria* sp. was not reported in studies by Ibrahim *et al.* (2014), Al Momani *et al*. (2017) and Parveen *et al.* (2017). The low frequency of occurrence observed may be due to the low oxygen tolerance of *Neisseria* sp.

Drug resistance continues to threaten our ability to treat common infectious diseases. In the present study, all the isolated Gram-positive bacteria showed highest sensitivity to gentamycin (100%) out of the 8 antibiotics tested. However, they all exhibited resistance to cloxacillin, ceftadizime and erythromycin (100%). The present study is in close agreement with the findings of Jain and Yadav (2017) and Oloso *et al.* (2018), who reported that isolated bacteria from eggs were *s*usceptible to gentamycin whereas demonstrated high resistance to cephalosporin, macrolides and other beta lactams. This could be attributed to their indiscriminate, haphazard and repeated use in poultry farms in different parts of the World including Nigeria (Islam *et al.*, 2018).

Islam *et al*. (2018) and Jain and Yadav (2017) found, in agreement with our results, no ciprofloxacin resistant enterobacteria, but, unlike their results, Gram positive bacteria from this study showed varying resistance to ciprofloxacin. However, the resistance of isolated *E. coli, Salmonella* sp. and *Shigella* sp. to augmentin and amoxicillin suggests the possible abuse and widespread of resistance to these agents in the study area.

Finally, result analysis revealed all the isolated bacteria from eggshells were resistance to more than one antibiotic in a given class except *Neisseria* sp. This is dangerous to public health as potential transfer of these bacteria to the consumer is possible. In addition, MAR index of all isolates were greater than 0.2.

This indicates that multidrug-resistant bacteria isolated from retailed eggs in the three major markets in Minna are from a high-risk source, possibly human or animal faeces, where indiscriminate use of antibiotics might have aided their development of resistance towards several antibiotics.

Conclusions

The eggshells of retailed eggs in the three major markets in Minna were contaminated with antibiotic resistant *Clostridium* sp., *Enterococcus* sp., *S. aureus, S. pyogenes, E. coli, Neisseria* sp., *Salmonella* sp. and *Shigella* sp. The transfer to consumers through contaminated eggs pose greater risk to public health.

Recommendations

The use of antibiotics in poultry farms should be reduced. Good hygiene practices should be employed in the collection, storage and processing of eggs.

Conflict of Interest:

The authors declare no conflicting interest in the publication No funding was obtained from any funding agency but the research was fully funded by the authors.

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