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Occurrence of *Salmonella* Spp. and *Escherichia coli* in Table Eggs in Kaduna and Zaria, Nigeria

[1]U. Akpabio, [2]J.K.P. Kwaga, [2]J. Kabir and [3]V.J. Umoh

[1]Department of Veterinary Public Health and Preventive Medicine,
Michael Okpara University of Agriculture Umudike, Nigeria
[2]Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, Nigeria
[3]Department of Microbiology, Akwa-Ibom State University, Ikot - Akpadem, Nigeria

Abstract: This study was carried out to determine the public health risks posed by table eggs due to contamination with *Salmonella* and level of contamination of table eggs with aerobic and coliform bacteria. A total of 3820 pooled egg, 114 cloacal and 140 ovarian follicle samples were tested for *Salmonella*. Also 100 pooled egg samples were analyzed for total aerobic counts, total coliform counts and *Escherichia coli.* Of the 100 pools, 3 were positive for *E. coli* for which antimicrobial susceptibilities pattern were tested using a panel of 12 antimicrobial agents. There was no significant difference ($P > 0.05$) in the mean total aerobic counts and coliform counts. There was a negative correlation of the total aerobic and coliform counts ($r = -0.028$), total aerobic counts and antibiotics usage ($r = -0.05472$) as well as correlation of coliform counts and antibiotics use ($r = -0.187$). The bacterial isolates were resistant to multiple antibiotics.

Key wods: Eggs · Contamination · Public Health · *Salmonella* · *Escherichia Coli*

INTRODUCTION

Salmonella is one of the major bacterial organism that cause foodborne infections in humans worldwide [1, 2]. Poultry and poultry products that are contaminated with these organisms have been implicated as a major source of human salmonellosis [2, 3]. Poultry that are infected with *Salmonella* but show no clinical illness maybe important in the spread of infection between flocks and as a source of food poisoning in humans [4, 5]. *Salmonella* Typhumurium and *Salmonella* Enteritidis are the commonest cause of non- typhoidal salmonellosis in humans [1, 6]. The global spread of *Salmonella enterica* serovar Enteritidis in chickens [7, 8] has resulted in an international food poisoning pandemic [9, 10, 11], contaminated eggs and egg products remain the main source of infection [12].

Although *Salmonella* is ubiquitous, the primary reservoir is the intestinal tract of animals and the colonisation is favoured by intensive animal production. Poultry products are frequent vehicles in the transmission of *Salmonella*, dominating other foods of animal origin as potential source of infection [13, 14].

Salmonella is transmitted both vertically and horizontally, thereby causing problems at all levels of poultry breeding and production. Infected hens can shed live bacteria into eggs, contaminating both table eggs and chicks. Horizontal transmission of *Salmonella* can take place from even a very small number of shedders [15, 16].

The widespread use of antimicrobial agents in food animal production has contributed to the occurrence of resistant bacteria in animals, including zoonotic pathogens, which can be transmitted to humans via the food chain [17- 20]. Since most *Salmonella* infections are acquired from ingestion of contaminated foods of animal origin, a likely cause for the increasing prevalence of antimicrobial-resistant *Salmonella* is the use of antimicrobial agents in food animals [21]. In recent years, the occurrence of this disease in humans has increased. NIAID, 2005 [22-24] reported that most infections have been attributed to consumption of poultry meat and eggs. In addition to *Salmonella* Enteritidis and *Salmonella* Typhimurium, many other serovars have been associated with food borne infections from contaminated poultry meat [25]. Numerous outbreaks of salmonellosis have

Corresponding Author: U. Akpabio, Department of Veterinary Public Health and Preventive Medicine, Michael Okpara University of Agriculture Umudike.

been reported usually involving the consumption of raw or undercooked eggs [26-28]. In eggs, a strain of major concern is *S. enterica subsp enterica* serotype Enteritidis [29].

Coliforms were historically used as indicator microorganisms to serve as a measure of faecal contamination and thus potentially of the presence of enteric pathogens in foods. Although coliform bacteria themselves are not pathogenic, their presence indicates possible faecal contamination and the corresponding presence of intestinal pathogens responsible for a variety of diseases. Within the coliforms *Escherichia coli* is of interest since when present in foods it indicates direct or indirect faecal contamination. It is also an indicator of the possible presence of enteric pathogens in water, shellfish, dairy products and other foods. High counts of *E. coli* and total coliforms in foods usually indicate lack of hygiene in handling and production operations, inadequate storage and post-process contamination [30, 31].

E. coli is a bacterium whose natural habitat is the enteric tract of humans and warm-blooded animals [32]. *E. coli* and total coliform enumeration are used as food-quality parameters. *E. coli* typically colonizes the infant gastrointestinal tract within hours of life and, thereafter, *E. coli* and the host derive mutual benefit [33]. *E. coli* usually remains harmlessly confined to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal "nonpathogenic" strains of *E. coli* can cause infection. Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can disseminate throughout the body. Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis and (iii) enteric/diarrhoeal disease [34].

E. coli is the most important agent causing secondary bacterial infection in poultry and may also be a primary pathogen [35]. Colibacillosis is the most frequently reported disease in surveys of poultry diseases or condemnations at processing [36]. It refers to any localized or systemic infection caused entirely or partly by avian pathogenic *Escherichia coli* (APEC). The most common form of colibacillosis is colisepticaemia and is responsible for significant economic losses in the poultry industry in most parts of the world [37]. In the past few years, both the incidence and severity of colibacillosis have increased rapidly and current trends indicate that it is likely to continue and become an even greater problem in the poultry industry [38, 39].

The aim of this study was to determine the public health risks posed by table eggs due to contamination with *Salmonella* and level of contamination of table eggs with aerobic and coliform bacteria.

MATERIALS AND METHODS

The following materials were used in this study;

Tetrathionate broth Base CM0029 (OXOID), xylose lysine deoxycholate Agar VM603087618 (MERCK), Nutrient Agar, Triple Sugar Iron Agar (OXOID), Urea Agar Base Cm0053 (OXOID), M.R.V.P CM0043 (OXOID), MacConkey agar, Sterile normal saline, Stomacher Blender 400 (Model No. BA 6021), Distilled Water, Sterile swab sticks, cotton wool, bijou bottles, universal bottles, Petri dishes, Pasteur pipette, polythene bags, peptone water Microbact Reagent Set D MB1082A (OXOID), Microbact Reagent Nitrate A MB0186A (OXOID), Microbact Reagent Nitrate B MB0187A, Microbact GNB 24E, Salmonella Test kit (OXOID DR1108A), Refrigerator, Test Tubes, adonitol, mannitol, arabinose, rhamnose, dulcitol, meso-inositol, sucrose, lactose, glucose, d-sorbitol, salicin, raffinose, arginine, lysine, ornithine, Oxoid antibiotics disks and Oxoid antibiotics dispenser.

Study Area: Sampling for this study covered four Local Government Areas (LGAs) in Kaduna State which includes Kaduna North, Kaduna South, Sabon -Gari and Zaria.

Geographical Location of Kaduna State: Kaduna State is located within the semi-arid and sub-humid regions of the north western zone of Nigeria. It lies between longitude E006.5°-E008.6° (East of Greenwich meridian) and latitude N09.2°-N11.3° (North of equator).

Sampling: The number of farms in each Local Government Area to be sampled were not equal, so proportionate probability sampling technique was used i.e. 25% proportion of registered farms in the each of the LGAs were selected for sampling. Major commercial egg depots were also sampled from each LGAs making a total of eight collection. There were 90 registered farms in these LGAs and this gave a total of 23 farms to be sampled. Two major slaughter slabs were visited in Kaduna and Zaria.

Cloacal swabs were collected from ten birds using a sterile swab in each of the farms visited and these were placed in sterile universal bottles containing 20 ml peptone water. Ten eggs were also bought from each farm; all the egg samples from each of the farms and

markets were pooled in tens to form a sample. Ovarian follicles were collected from slaughtered hens using a sterile polythene bag.

Two major commercial egg depots were also sampled from each LGAs making a total of eight collection points where eggs were obtained from major egg marketers. Sampling was carried out every two weeks for three months. All the egg samples from each of the farms were pooled in tens to form a sample. All the samples were transported to the Bacterial Zoonoses Laboratory of Department of Veterinary Public Health and Preventive Medicine in sterile polythene bags.

Sample Processing for *Salmonella* Isolation: The egg shells were disinfected with 70% ethanol, the taper end of each egg was broken aseptically using a thumb forceps. The contents of the pool of ten eggs i.e. egg yolk and egg white were emptied into a sterile polythene bag and homogenized using a stomacher. 10 ml of the homogenate was inoculated into 90 ml of tetrathionate broth enrichment medium and incubated at 37°C for 24 hours.

Cloacal swab samples in peptone water which served as pre-enrichment medium were incubated at 37°C for 24 hours. 1ml of the pre-enrichment broth was inoculated into 9ml of tetrathionate broth and incubated at 37°C for 24 hours.

Ovarian follicles were weighed and 1g of each was decontaminated by dipping into boiling water for 5-10 seconds. They were then chopped into tiny pieces using sterile scissors and forceps and each added to 9 ml tetrathionate broth and incubated at 37°C for 24 hours.

Selective Plating and Identification of *Salmonella* Isolates: A loopful of the inoculum from each enrichment broth above was transferred to the selective agar. In this study xylose lysine deoxycholate agar was used. The inoculum was streaked on the agar and incubated at 37°C for 24 hours. On this medium, *Salmonella* colonies appear as pinkish colonies with or without dark center. All non-lactose fermenting organisms were picked from the plate and inoculated into nutrient agar slants, incubated at 37°C for 24hours and stored in a refrigerator at 4°C pending further studies. The stored slants were considered to be presumptive isolates.

Preliminary and Complete Biochemical Testing: All agar based media and substrates were prepared according to the Manufacturer's instructions. Sugars and amino acids were also prepared using standard procedures. The purity of the isolates were ascertained by plating on xylose lysine deoxycholate agar before stabbing and streaking on triple sugar iron and urease agar slants.

Colonies were picked and streaked on triple sugar iron agar and urease agar slopes while the butts of Triple Sugar Iron agar were stab- inoculated. These were incubated at 37°C for 24hours. *Salmonella* suspected organisms were expected to give alkaline over acid reaction in TSI agar slants, with or without H_2S and gas respectively. A positive urease test was indicated by pinkish discolouration of the slant.

Complete biochemical testing of the isolates were carried out using the following sugars; salicin, mannitol, rhamnose, meso-inositol, adonitol, arabinose, raffinose, glucose, sucrose, lactose, sorbitol and dulcitol. The following amino acids were also tested for decarboxylation, arginine, lysine and ornithine.

Sugar Fermentation and Amino Acid Utilization Test: The sugars were tested by using standard procedures with bromothymol blue as the indicator. The amino acids were prepared at 1% concentration and 1ml of sterile paraffin added to each tube. Bromocresol-purple and phenol red were used as indicator for amino acid utilization. These were inoculated with the test organisms and incubated at 37°C for four days and checked for colour change.

Changes in colour from green to yellow for the sugars indicated a positive reaction, while the green colour indicates a negative reaction. Amino acid utilization tests were performed using the modified Falkows methods. Using sterile wire loop, tubes containing 1% concentration of lysine, ornithine and arginine broth with 1ml sterile paraffin added to each tube were inoculated with the test organisms and incubated at 37°C for four days and examined daily for colour changes from yellow to purple for positive test results.

Identification of *Salmonella* Using Microbact GNB 24E: Prior to testing, all isolates were streaked on xylose lysine deoxycholate agar plates and incubated at 37°C for 24 hours. Sterile normal saline was prepared and 5 ml dispensed into each test-tube. Using a sterile loop 1-3 colonies of the culture was picked and emulsified in the 5 ml sterile saline, this was mixed thoroughly to prepare a homogenous suspension to yield a turbidity equivalent to 0.5 McFarland's standard. The wells of the individual substrate sets was exposed by cutting the end tag of the sealing strip and slowly peeled backward. The plate was

placed in a holding tray and using a sterile Pasteur pipette, four drops of the bacterial suspension was added to each well set.

Using a sterile pipette the substrates underlined on the holding tray were overlayed with mineral oil i.e. wells 1, 2, 3, 20 and 24. The inoculated rows were resealed with the adhesive seal. The specimen number was written on the end tag with a marker pen. The plate was incubated at 37°C for 24 hours. The tray was removed from the incubator after 24 hours, the adhesive seal peeled, Nitrate, Kovacs, Voges-Proskauer and Tryptophan Deaminase reagents were added to wells 7, 8, 10 and 12 respectively. The results were interpreted as stipulated by the Manufacturerers, using the supplied software version Microbact™ 2000 Identification package V2.03 (Windows™).

Identification of *Salmonella* Strains: Isolates suspected to be *Salmonella* were serologically tested using *Salmonella* polyvalent 'O' group A-Z antiserum latex kit according to the instruction of the Manufacturer (OXOID). All isolates were streaked on xylose lysine deoxycholate agar plates and incubated at 37°C for 24hours. Latex reagents were brought to room temperature, one drop of the test latex reagent was dispensed onto a circle on the reaction card and a drop of saline was placed on the circle distant from the latex. Using a loop, a portion of the colony of presumptive *Salmonella* spp on xylose lysine deoxycholate agar plates was emulsified in the saline drop on a portion of the circle on the card. The test latex and the resulting smooth suspension were mixed together and spread to cover the reaction card using the loop. The card was then rocked in a circular motion observing for agglutination within two minutes.

Isolation of *E. coli* from Eggs: One hundred pooled egg samples were collected from the farms and tested for *E. coli*. The egg shells were disinfected with 70% ethanol, the taper end of each egg was broken aseptically using a thumb forceps. The contents of the pool of ten eggs i.e. egg yolk and egg white were emptied into a sterile polythene bag and homogenized using a stomacher. The homogenate was serially diluted by ten (10) fold into universal bottles containing sterile normal saline i.e.1ml of the homogenate was inoculated into 9ml sterile normal saline.

Total Aerobic Counts: The diluted sample (0.1ml) was aseptically transferred into nutrient agar plates. The inoculum was spread using an aseptic glass rod spreader. The plates were incubated at 37°C for 24hrs. Total aerobic counts were determined and recorded from nutrient agar plates.

Total Coliform Counts: The diluted sample (0.1ml) was aseptically transferred into MacConkey agar plates. The inoculums was spread using an aseptic glass rod spreader. The plates were incubated at 37°C for 24 hours. Total coliform counts were made from the MacConkey agar plates. Lactose fermenting organisms from the MacConkey agar plates were identified and picked from the plates and inoculated into nutrient agar slants incubated at 37°C for 24 hours and thereafter stored in a refrigerator at 4°C pending further studies.

Biochemical Test: Biochemical tests were performed to confirm E. coli using Gram staining, Catalase test, Indole, Methyl red, Voges- Proskauer test, Nitrate reduction, Urease production, Simmon's citrate agar and various sugar fermentation tests.

Antimicrobial Susceptibility Testing of Bacterial Isolates: This was performed using a panel of 12 antimicrobial agents by disk diffusion method following CLSI guidelines (CLSI 2002) and cultured on Mueller Hinton agar.

Sterile nutrient broth was prepared according to the Manufacturer's instruction; the test isolates were inoculated into the broth and incubated at 37°C for 24 hrs. The broth culture was adjusted with sterile saline to obtain turbidity optically comparable to 0.5 McFarland standards. Mueller Hinton agar was inoculated with 0.1ml of the nutrient broth culture and spread over the entire sterile agar surface. The drug impregnated disks used (OXOID) contained Sulphamethoxazole/trimethoprim (25µg), Lincomycin (10µg), Nitrofurantoin (50µg), Gentamicin (10µg), Amoxicillin/clavulanic acid (50µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Kanamycin (30µg), Ampicillin (10µg), Streptomycin (10µg), Tetracycline (30µg) and Penicillin G(10units) were placed individually on the surface of inoculated agar plates using a dispenser (OXOID) and incubated at 37°C for 18hrs. The zones of inhibition were measured to the nearest millimeter using a ruler.

RESULTS

Isolation of *Salmonella* from Egg Samples: A total of 3820 pooled egg samples were analyzed for the presence of *Salmonella*. There were 12 suspects, consisting of 3 egg samples from Kaduna north LGA, 1 sample from

Table 1: Isolation of Salmonella from egg samples in different LGAs

LGA SAMPLED	No of Pooled egg Samples	No Positive	% Positive
Kaduna North	955	3	0.3
Kaduna South	955	1	0.1
Sabon Gari	955	8	0.8
Zaria	955	0	0

Table 2: Total aerobic and coliform counts in different LGAs

LGA Sampled	No of Pooled Egg samples	No Positive	% Positive
Kaduna North	25	12	48
Kaduna South	25	14	56
Sabon-gari	25	16	64
Zaria	25	15	15

Table 3: Mean of total aerobic and coliform counts

	Mean (± SEM)	Significant
TAC	14.77 ± 3.49	P > 0.05
TCC	18.43 ± 5.02	P > 0.05

TAC = Total aerobic counts
TCC = Total coliform counts

Table 4: Correlation between total aerobic and total coliform counts

Variable	Pearson r
Total aerobic and coliform counts	-0.028

Table 5: Biochemical characterization of *E. coli*

Biochemical Test	Reaction
Indole Production	+
Methyl Red	+
Voges Proskauer	–
Simmon's Citrate	–
Urease	–
Nitrate Reduction	+
Catalase	+
Lactose fermentation	+
Mannitol	+
Lactose	+
Salicin	+
Sucrose	+
Glucose	+

Table 6: Antimicrobial drug susceptibilities

Sample ID	Isolate	Location of Farm	Antibiotics Used	Resistance profile
EX7	*Serratia plymuthica*	Samaru	ENR, TE	AMC, AMP, MY P
EX8	*Serratia liquefaciens*	Samaru	C, CIP, TE	AMP, MY, P, S, TE
EX10	*Enterobacter agglomerans*	Samaru	C, ENR, TE	AMC, AMP, MY, P, TE
KS3E2	*Escherichia coli*	Kaduna South	TE	C, MY, P, TE
KN3E2	*Escherichia coli*	Kaduna North	TE	C, MY, TE
OKE2	*Escherichia coli*	Samaru	C, TE	C, MY, P, TE

Enrofloxacin (ENR), Tetracycline (TE), Chloramphenicol (C), Amoxicillin/Clavulanic Acid, (AMC), Ampicillin (AMP), Lincomycin (MY), Penicillin G (P), Ciprofloxacin (CIP), Streptomycin (S)

Kaduna south LGA and 8 from Sabon-gari LGA. After carrying out the preliminary biochemical tests only 3 of the suspects from Sabon-gari LGA were negative on urease agar slant and produced the expected test result on TSI slants. The 3 suspects were subjected to complete biochemical tests, Microbact 24E tests and serological tests using *Salmonella* polyvalent antiserum (Oxoid). The microbact tests were recorded on the microbact chart

and identification of the organisms were carried out by the use of the microbact software. The software showed the probability percentage of the following organisms: *Serratia plymuthica* (51.71%), *Serratia liquifaciens* (62.09%), *Enterobacter agglomerans* (76.82%). The serological tests were also negative for the 3 suspects.

Isolation of *Salmonella* from Cloacal Swab and Ovarian Follicle Samples: A total of 114 cloacal swab and 140 ovarian follicle samples were analyzed for the presence of *Salmonella*. There were 21 suspects, 1 cloacal swab sample from Kaduna north LGA, 10 from Sabon-gari LGA, 2 from Zaria LGA and 8 ovarian follicle samples from Sabon-gari LGA. After the preliminary biochemical tests none of the 21 suspects yielded the expected reactions typical for *Salmonella* on urease though they showed the expected result on TSI slants.

Total Aerobic Counts, Total Coliform Counts and Isolation of *E. coli*: A total of 100 pooled eggs were tested from farms in the four LGAs. Twenty six pooled samples had aerobic growth and 21 had coliforms. *E. coli* were identified from the coliforms by subjecting the coliforms to preliminary and complete biochemical test. The result of the biochemical test showed that only three of the isolates were positive for *E. coli*.

There was no significant difference ($P > 0.05$) in the mean total aerobic counts and coliform count (Table 3). There was a negative correlation of the total aerobic and coliform counts ($r = -0.028$) (Table 4). There was a negative correlation of the total aerobic counts and antibiotics usage ($r = -0.05472$), correlation of coliform counts and antibiotics use was also negative ($r = -0.187$).

DISCUSSION

Feed additives are included in diets for poultry in order to increase production by improving nutrient availability. Antibiotics as feed additives act as growth promoters by preventing disease occurrence thus improving the efficiency of animal production. Antibiotics have been used in livestock farming for several decades in combating bacterial infections. The use of antibiotics as growth promoter in diets for layers has been discouraged. In the European Union as in many developed countries the inclusion of antibiotics in animal feed is forbidden because residues of these drugs may appear in eggs constituting potential health hazards to the consumers in addition to risks of promoting antibiotics resistance [40-42]. In this study 24% of the farms used feed additives; farms that compounded their feed locally reported the addition of antibiotics as feed additives. Also related to the use of antibiotic feed additives, the farms studied in this work were also found to be widely and frequently using antibiotics. In particular tetracycline, erythromycin, ciprofloxacin and chloramphenicol were misused in most of the farms surveyed. It was a common practice by farmers in the study area to administer drugs without consulting a veterinarian and the drug withdrawal period was not observed. The level of *Salmonella* contamination in table eggs was determined in this study. *Salmonella* was not isolated from eggs in this study; this may not be unconnected with the gross misuse of antibiotics in the study area. The use of antibiotics could have interfered with the isolation of *Salmonella* from cloacal swab due to intermittent bacterial excretion or antibiotics use in feed and water or as a prophylactic treatment [43] reported a similar finding; in which they also failed to isolate *Salmonella* from cloacal swabs that were also attributed to antibiotic suppression. Raufu *et al.* [44] reported 23% prevalence of *Salmonella* from cloacal swabs. Despite the fact that *Salmonella* may occur due to well-known factors especially the low level of biosecurity that was observed in the study area, it was still not isolated in this study.

In this study 21 of the isolates were positive for coliform and 26 were positive for aerobic bacterial growth. *E. coli*, *Serratia liquefaciens*, *Serratia plymuthica* and *Enterobacter agglomerans* were isolated although in very few numbers perhaps is an implication to the pressure of antibiotic use. The bacterial isolates were resistant to tetracycline, chloramphenicol, lincomycin, ampicillin, amoxicillin and clavulanic acid, streptomycin and penicillin G. These multiple resistance is an indication of the need for the control of excessive antibiotics use. When the intensity of antibiotics use was compared with the aerobic and coliform counts in the eggs tested in this study a negative correlation was seen suggesting that the more the antibiotic use, the less the bacterial counts in the eggs. Though not categorically determined, there is greater likelihood that the aerobic and coliform bacteria in these eggs would be largely antibiotic resistant.

The 3 *E. coli* isolates were all resistant to tetracycline, chloramphenicol and lincomycin. This is in contrast to the study of Shtylla *et al.* [45] in Albania who reported that the highest level of resistance was observed for erythromycin (100%), amoxicillin (99.1%) and tetracycline (96.07%). It is also in contrast with the report of Muhammad *et al.* [46] in Bangladesh who reported a resistance rate of 30% for chloramphenicol and 52% for

tetracycline from poultry and the report of Anyanwu *et al.* [47] in Jos who observed that *E. coli* were sensitive to chloramphenicol for samples collected from day old chicks. The present study is similar to the report of Okoli *et al.* [48] and Chah *et al.* [49] in Owerri who also recorded a hundred percent resistance to tetracycline, chloramphenicol, nitrofurantoin, cotrimoxazole and ampicillin and 93.3%, 90.0%, 70.0% and 60% resistance to ampicillin, tetracycline, chloramphenicol and nitrofurantoin respectively for isolates from commercial layers. These organisms may constitute enormous reservoirs of genes encoding resistance against these antibiotics and foci for continual spread of these mechanisms [50, 51]. Though the isolates were from different areas, multi drug resistance (MDR) was a common feature in these isolates, highlighting the fact that the resistance genes for these drugs may be linked on plasmids [52].

The eggs were contaminated with other organisms including *Serratia liquefaciens*, *Serratia plymuthica*, *Enterobacter agglomerans*.

CONCLUSION

The findings in this study illustrates the risks of occurrence of *Salmonella* in poultry production in Nigeria due to the low level of biosecurity and this is attributed to the high level of use of antibiotics in production.

This study also found that most poultry farmers were not observing strict biosecurity measures on their farms. *Salmonella* was not isolated from eggs, ovarian follicles and cloacal swabs in this study. The eggs were contaminated with other organisms including *Serratia liquefaciens*, *Serratia plymuthica*, *Enterobacter agglomerans*, *Klebsiella ozoanae, Enterobacter hafnei* and *Citrobacter freundii.*

Consumers of eggs from these farms are at risks of exposure to drug residues in view of the practice of the farmers in the study area on the use of antimicrobials.

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