Determination of Antibiotic Resistance of Salmonella and Molecular Confirmation of Drug Resistance Salmonella Species Isolated From Raw Chicken and Quail Eggs from Selected Farms in Jos, Nigeria

Dr. G.M. Gberikon  
Lecturer, Department of Microbiology, Federal University of Agriculture Makurdi, Nigeria  

Olabode, V.B  
Assistant Chief Medical Laboratory Scientist, Department of Central Diagnostic Laboratory Veterinary Research Institute, VOM, Plateau State, Nigeria  

Dr. I.O. Ogbonna  
Lecturer, Department of Microbiology, Federal University of Agriculture Makurdi, Nigeria

Abstract:  
Determination of antibiotic resistance of Salmonella and molecular confirmation of drug resistance Salmonella species isolated from raw chicken and quail eggs from selected poultry farms in Jos Local Government Area was carried out. A total of 360 egg samples were randomly collected, one hundred and eighty (180) each from quail and chicken respectively from three Local Government Areas namely; Jos north, Jos south and Jos east were sampled. The samples were examined for the presence of Salmonella species using standard microbiological and biochemical techniques. They were tested for antimicrobial susceptibility by disc diffusion method. Results showed that out of the 360 samples, only 3(1.7%) from chicken egg shells were positive for Salmonella. Positive result for Salmonella was not obtained from quail egg shells nor contents. Isolates from results were further subjected to susceptibility testing to eleven (11) commonly used antibiotics. Three (3) were 100% resistant to Streptomycin, Erthromycin in and Oxacillin. The isolates were also 67% resistant to Ampicillin, Ciprofloxacain and Chloramphenicol. All the three (3) isolates were successfully amplified using specific primers for PCR method. The results showed presence of Salmonella species in only chicken egg shells. Poultry eggs should be properly washed before consuming it raw so as to reduce infections by this organism.

Keywords: Chicken eggs, quail eggs, salmonella, antibiotics, egg shells, egg contents

1. Introduction  
Bacteria of the genus Salmonella are Gram negative, facultative anaerobic, non-spore forming, usually motile rods belonging to the Enterobacteriaceae which are associated with alimentary tracts of animal (Douglas et al., 2015). Some species of Salmonella (S. enterica) is zoonotic; that is it can be pass from animal to man through consumption of contaminated food (Douglas et al., 2015). Salmonella enterica associated with poultry products has constituted public health and economic issues worldwide. The emergence of antibiotic resistance Salmonella recovered from poultry products was heightened with the use of antibiotics in their feeds (Obafemi and Davis, 1986). Eggs are still considered to be an excellent source of chlorine and selenium and a good source of riboflavin. The protein found in eggs is highly digestible; the yolk contains vitamins A, D, E and K, folic acid, pantothenic acid and Zinc (Egg Nutrition Center, 2004). World over, consumption of eggs have gone up considering the nutritional importance of it to man. Antibiotic resistance is widespread and resistance has been elevated by world health organizations as one of the top health challenges. The escalating cases of antibiotic resistance has raised concerns that we are entering “post antibiotic era” meaning we might enter an era where there won’t be effective antibiotics to treat many life threatening infections ((Douglas et al., 2015). Investigation towards cubing antibiotic resistance by some species of Salmonella should be given utmost attention.

2. Materials and Methods  

2.1. Sample Size  
A total number of 370 sample size was determine using prevalence rate from previous studies(Mai et al., 2013) and the desired absolute precision with the formula:  
n=Z²Pq/d²Naing et al. (2006).  
n = desired sample size (when population is greater than 10,000)  
Z = Standard normal distribution of 95% confidence interval = 1.96.
P = Known prevalence of the infection.

d = allowable error which is taken at 5% = 0.05

q = 1.0 - p

Using the formula Naing et al., (2006)

\[ q = \frac{p}{1-p} \]

Attrition rate = 10% of total sample i.e. 33.7

\[ = 337 \]

\[ = 370 \]

2.2. Statistical Analysis

Data was analyzed using the SPSS version 20 computer statistical software package. Questionnaire was administered and treated, and cross tabulations were done to examine relationship between categorical variables. The Chi-square test was used to compare differences between proportions. The statistical analysis was set at 5% level of significance (i.e. \( p < 0.05 \)).

2.3. Sample Collection

A total number of 360 sampled eggs were collected, 180 samples for chicken and 180 samples for quail. Samples were randomly collected from three (3) different locations in Jos town namely; Jos south, Jos north and Jos east.

2.4. Packaging for Laboratory Analysis

Chicken and quail eggs were collected in separate sterile plastic bags each, egg shell surfaces were swabbed with sterile swab stick and placed into buffered peptone water (BPW), to avoid dryness of the swab.

2.5. Sample Transport

All samples were placed in sterile plastic bags and then packaged in an ice box and transported immediately to Microbiology unit of Central Diagnostic Laboratory, Department of National Veterinary Research Institute Vom, and Plateau State Nigeria.

2.6. Sample Processing

All samples were processed according to standard guidelines of detecting Salmonella both in the egg shell and internal content by International Standard Organization (ISO): 6579, (2012) and Office International des Epizooties (OIE), (2012).

2.7. Media Used for Inoculation and Isolation

All the media (Nutrient Agar, (NA), Buffer Peptone water (BPW), Rappaport-vassiliadis broth (RVB), Xylose Lysine Desoxycholate Citrate Agar (XLD), Deoxycholate Citrate Agar (DCA)) used for inoculation and isolation were all prepared according to manufacturer’s standards as adopted by Cheesebrough (2001).

2.7. Swabs from Surface of Egg Shell

Surface swabs from egg shells collected was directly incubated in 9ml BPW in screw capped bottles and then incubated at 37°C for 24hrs for pre-enrichment. About 1ml of the pre-enrichment broth was transferred into tubes containing 10ml RVB, and then sub-cultured by streaking onto DCA and XLD agar. The sub-cultured plates were incubated at 37°C for 24hrs (Suresh et al., 2006; OIE, 2012).

2.8. Egg Internal Content

Eggs from the sterile plastic bag were aseptically opened with sterile scissors and the egg shell aseptically broken and the content from each egg were homogenized in a glass flask. Exactly 1ml of the homogenized egg was transferred in to 9ml of buffered peptone water (BPW) (Pre-enrichment broth) and incubated at 37°C for 24hrs.

2.9. Biochemical Test for Identification of Isolates

Gram staining, Sugar fermentation, motility, indole, oxidase, catalase tests, Methyl red and Voges-Proskauer test, Citrate utilization test, Urease test, Triple sugar iron test were all carried out adopting methods of Cheesebrough (2001) for identification of isolates.

2.10. Serotyping

Cultures of organisms from a pure culture identified as Salmonella by biochemical tests were serotype. The serological identification of Salmonella species was done using polyvalent Salmonella H antisera and Salmonella O antisera (Oxoid, UK). Three to five colonies of isolate were suspended in 0.5ml normal saline used as antigenic suspension. One drop of the polyvalent antiserum and normal saline was placed on a clean glass slide divided into parts with glass pencil as control. A drop of the antigenic suspension was placed to the antiserum and normal saline on another part of the glass slide. The suspension was mixed by tilting the glass slide back and forth for one minute. Big agglutination within one minute was observed and recorded as positive. Delayed or weak agglutination was recorded as negative. Standard positive and negative controls were also run at the same time (Cowan and Steel, 1993).
2.11. Antibiotic Sensitivity Testing

Antibiotic susceptibility test was carried out by the disc diffusion method. This was done by inoculating pure culture identified as Salmonella isolate using sterile wire loop to pick 2-3 colonies and emulsify in 5ml of sterile normal saline. About 0.5ml of the broth was further transferred into 5ml of Muellar-hinton broth for 16-18hrs at 37°C before swabbing using cotton swab onto dried surface plate of sterile Mueller Hinton Agar (MHA). After 15min of pre- diffusion time, antibiotic disc were placed on the seeded agar surface separated from each other to avoid overlapping of inhibition zones. The plates were incubated at 37°C for 24hrs. The diameter of inhibition zones were measured with a ruler and compared with a zone interpretation chart. The antibiotic resistance was noted using modification of the kirby-Bauer disk (Cheesebrough, 2001).

2.12. DNA Extraction of Isolated Salmonella

This was carried out using boiling method by the techniques of Sunar et al. (2014). Two hundred (200) µl of culture was incubated at 95°C for 5 minutes, it was immediately put on ice for 10 minutes and subsequently centrifuged at 10,000 rpm for 3 minutes. The supernatant was stored at -20°C for PCR amplification.

2.13. Primers Set and Polymerase Chain Reaction Amplification

Salmonella specific primers, based on the invA gene of Salmonella was used. Forward: 5’ GTG AAA TTA TCG CCA GTC TCG GGC AA3’ and Reverse: 5’ TCA TCG CAC CGTCAA AGG AAC C3’.

2.14. PCR Amplification

PCR was carried out in a 2X master mix composition of Taq 2X Master Mix (New England Biolabs™) containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, and 0.2 mM of each dNTPs. The mix was carried out in a 25µl total volume of enzyme mix per sample. Amplification was conducted in applied biosystems Thermo Cycler AB 700.

The cycle conditions used is as follows: an initial incubation at 94°C for 60 seconds, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 64°C for 30 seconds, and elongation at 72°C for 30 seconds, and finally 7 minutes of extension at 72°C.

2.15. Detection Using Gel Electrophoresis

Measurement of 1.5 % w/v of agarose gel was prepared using Tris- borate-EDTA (TBE) buffer. Ten (10) micron liter of ethidium bromide was added and poured into the casting tray with combs placed at each end and allowed to polymerize. The tray was gently placed in TBE electrophoretic tank. Each sample was placed in each separate sample wells and O’ Gene Ruler 100bps DNA ladder at the beginning and end of each well. After loading the samples in each well, the electrophoretic tank was connected to the power source and run at 140volts, 400Amperes for 35 minutes after which results were viewed using a gel imaging documentation apparatus (MB Fermentase USA).

3. Results and Discussion

| LGA           | Quail Eggs | Chicken Eggs | Total |
|---------------|------------|--------------|-------|
|               | Number of samples Examined | Number Positive (%) | Number of samples Examined | Number Positive (%) | Number of samples Examined | Number Positive (%) |
| Jos east      | 60         | 0(0.0)       | 60    | 0(0.0)       | 120   | 0(0.0)       |
| Jos north     | 60         | 0(0.0)       | 60    | 0(0.0)       | 120   | 0(0.0)       |
| Jos south     | 60         | 0(0.0)       | 60    | 3(5.0)       | 120   | 3(2.5)       |
| TOTAL         | 180        | 0(0.0)       | 180   | 3(1.7)       | 360   | 3(0.9)       |

Table 1: Prevalence of Salmonella Species Isolated From Quail and Chicken Eggs in the Three L.G.A Sampled

\( \chi^2 = 4.336, P< 0.005, \text{Df}=2 \)

| Test                  | Results          |
|-----------------------|------------------|
| Polyvalent O Antisera | Agglutination (+) |
| Polyvalent H Antisera | No agglutination (-) |

Table 2: Serological Identification of Salmonella Isolates (Slide Method)
| Antibiotic                        | Sensitive (%) Isolates | Intermediate Isolates | Resistant Isolates |
|----------------------------------|------------------------|-----------------------|--------------------|
|                                  | Number | %   | Number | %   | Number | %    |
| Amoxicillin/Clavulanic acid      | 2      | 66.7| 1      | 33.3| 0      | 0.00 |
| Ampicillin                       | 1      | 33.3| 0      | 0.00| 2      | 66.7 |
| Oxacillin                        | 0      | 0.00| 0      | 0.00| 3      | 100  |
| Chloramphenicol                  | 1      | 33.3| 0      | 0.00| 2      | 66.7 |
| Ciprofloxacin                    | 0      | 0.00| 1      | 33.3| 2      | 66.7 |
| Gentamycin                       | 2      | 66.7| 0      | 0.00| 1      | 33.3 |
| Tylosin                          | 2      | 66.7| 0      | 0.00| 1      | 33.3 |
| Streptomycin                     | 0      | 0.00| 0      | 0.00| 3      | 100  |
| Tetramycin                       | 1      | 33.3| 2      | 66.7| 0      | 0.00 |
| Trymethoprim/sulfamethoxazole    | 2      | 66.7| 1      | 33.3| 0      | 0.00 |
| Erythromycin                     | 0      | 0.00| 0      | 0.00| 3      | 100  |

Table 3: Antibacterial Resistant Profile of Salmonella Isolates (N=3)

4. Discussion

Results from this study showed difference in the isolation rate from the three (3) Local Government Areas surveyed. The isolates from Jos South were higher compared to Jos North and Jos East. Bata et al. (2016) also reported high isolation rate in Jos south compared to Jos north and Jos East. The difference in the distribution of isolates may be due to social-demography difference and other bio-security practices in the study areas. It is important to note that even though the serotype isolated in this study was S. gallinarum which is host specific, was isolated from the shell of chicken eggs and none isolated in egg contents. The antibiogram profile of the Salmonella isolates in this study revealed that, all the isolates acquired resistance to 2-3 antibiotics. Multidrug resistant Salmonella in poultry and poultry products has been reported worldwide (Okoli et al., 2006). Antibiotic resistance to Salmonella species has been reported also in poultry eggs (Jelalu et al., 2016) and in commercial chickens (Min et al., 2010; Fasure et al., 2012). In this study, antibiogram profile revealed that all the Salmonella isolates were 100% resistant to streptomycin erythromycin and oxacillin. Highest resistance was 67% from ampicillin, chloramphenicol and ciprofloxacin. The least resistant was to tylosin and gentamycin.
This is partially in agreement with the findings reported by Bata et al. (2016) in Jos, Plateau State, who reported that Salmonella isolates were completely resistant to tetracycline, erythromycin, neomycin and oxacillin. One of the studies in Spain reported high percentages of resistance of Salmonella isolated to neomycin, tetracycline and streptomycin (Carraminana et al., 2004). This resistance might be the result of indiscriminate use of these antibiotics as prophylaxis, growth promoter or treatment. In a study by Ayale et al. (2011) the sensitivity of the Salmonella isolates was 14.2% to tetracycline, 28.6% to chloramphenicol, which is in agreement with the present study with highest susceptibility level detected in tetracycline (70%) chloramphenicol and trimethoprin/sulfamethoxazole. Drug resistance in food borne pathogens is the consequence of indiscriminate use of antimicrobial drugs in food producing animals like in poultry birds (Threlfall et al., 2000). To combat bacterial contamination, antibiotics are used in food animals. Of great concern is the high levels of antibiotic resistance as a result of the usage in food animals especially poultry which could predispose consumers to risks of antibiotic resistant bacterial infections (Okeke et al., 2005). The high antimicrobial susceptibility test result of Salmonella isolates in this study may also be as a result of antibiotics incorporated into feeds as supplements and growth promoters (Murugkar et al., 2005). Most of the poultry farms visited uses commercially made poultry feeds in which drugs had been incorporated directly from the mills. The lack of restriction of access by feed millers may indirectly expose the birds to resistance. The general high resistance against streptomycin, erythromycin, oxacillin, ampicillin and ciprofloxacin in this study also reflects high usage of the drugs by the farmers since they are inexpensive first line broad spectrum that are always available (Okeke et al., 2000). The accessibility and availability of these drugs probably develops into misuse and ineffective to the treatment of Salmonella diseases. Likewise, the low resistance observed against the other drugs could be due to its infrequent usage. Salmonella isolates processed by PCR in this study produced bands with amplicon size of 284bp. This corroborates with the work done by Nwiyi et al. (2016) and Anejo-Okopi et al. (2016). The outcome of the PCR result from the 3 isolates was 3/3 (100%) compared to the phenotypic method by culture 3/360 (0.8%) used. This result suggests that the use of invA gene by PCR is fast, more sensitive and specific than the conventional methods, and it will be a good alternative method for the detection of Salmonella species in food and clinical samples (Mamman et al., 2014). The difference in amplification size and difference in primer type used could be associated with the sensitivity of the PCR result due to the PCR employed in this study compared with results of other studies which reported higher sensitivity (Dione et al., 2011; Shanmugasamy et al., 2011).

5. Conclusion

It was concluded that the serotype isolated in this study was S.gallinarum which is host specific and was isolated from the shells of chicken eggs and none was isolated in egg contents. The antibiogram profile of Salmonella isolates in this study showed that, all the isolates acquired resistance to 2-3 antibiotics. The antibacterial susceptibility test of the Salmonella isolates in this study revealed that the isolates had acquired resistance to multi drugs.

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