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Molecular Detection of *Salmonella* Isolated from Poultry Farms in Abia State Southeast Nigeria

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A B S T R A C T

The study was conducted in six local government areas of Abia State, and this includes Aba north and south, Umuahia south and Ikwuano, Ohafia and Bendel local government areas and three senatorial zones that make up the State, namely, Abia south, Abia central and Abia north respectively. The aim of the study was to detect the presence of *Salmonella* isolated from poultry farms using molecular method. A total of 1420 samples were collected comprising 800 eggs, 420 clocal swabs and 200 poultry litter. The samples were processed in the veterinary microbiology laboratory. Universal primer set specific for genus *Salmonella* 16SrDNA 341F (5-CCTACGGGAGGCAGCAG-3 and 907R5-CCGTCATTT CCTTTRAGTTT-3) was used. The result show that a total of 28 *Salmonella* were isolated with egg, cloacal swab and litter presenting 18, 7 and 3 *Salmonella* distribution respectively and isolation rate of 1.97%. There were no significant association ($p > 0.05$) between rate of isolation and senatorial zone. The molecular study using agarose gel of amplification products shows that there was detection of *Salmonella species* with bands at 200bp.

Keywords

Salmonella, Poultry Farms, Avian Salmonellosis

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Introduction

Salmonellae are Gram-negative, short pump rods, non-spore forming, non-capsulated, aerobic and facultative anaerobic organisms and classified under the family enterobactericeae (OIE Manual, 2006). Avian salmonellosiss is the disease of birds caused by members of the genus *Salmonella* with particular reference to *Salmonella pullorium* and *Salmonella gallinarum* (Jordan, 1990). Disease caused by *Salmonella* infections is most common in Chicks under 2 weeks of age and is rarely seen in birds of 4 weeks of age for pullorum disease while fowl typhoid occurs among adult birds (Khan *et al.,* 1998). The morbidity and mortality vary considerably and deaths are usually less than 20% of the affected group but in exceptional cases can approach 100% (Lulful-Kabir, 2010).

The clinical signs as described by Freitas-Neto *et al.,* (2007) are depression, weakness, anorexia and dropping wings. Others are drop in egg production, prostration and apathy (Ezema *et al.*, 2009). Confirmation

of the diagnosis is by isolation and identification of the causal agent. In chick dying in the septicemia phase, salmonellae can be isolated directly from the liver, gall bladder or yolk sac, but the intestines and particularly the caecal contents are the most rewarding site to culture (OIE, Manual, 2004).

There are different sources *Salmonella* can invade farms, *Salmonella* infection can be from faecal contamination of eggs, introduction of *Salmonella* into a country via importation of live poultry or hatchable eggs and ineffective vaccination using live *Salmonella* vaccine (Bensink and Botham, 1983). Rats and mice are documented source of *Salmonella* and are attracted to the Poultry house by abundance of easily accessible food (Pomery and Nagaraja, 1991). Domestic flies and bettles are both capable of transmitting salmonellae and infection can persist through the insects from one generation to another via eggs and larva (Bustian and Aize, 2007). There has been several molecular methods of characterization of *Salmonella* with each method having merits and demerit (Sabat *et al.,* 2003). These include; Polymerase Chain Reaction (PCR), Pulsed-Field Gel Electrophoresis (PFGE) and Random Amplification of Polymorphic DNA (RAPD). Polymerase chain reaction (PCR) is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the other template strand. Since DNA polymerase can add a nucleotide only onto a pre-existing 3'OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify (NCBI, 2014).

The Pulse-Field Gel Electrophoresis is a standard molecular typing method used to analyze centre to centre transmission and have been successfully used in large scale microbiological and epidemiological investigations (Mac Dougal *et al.,* 2004). The use of Random Amplification of polymorphic DNA (RAPD) is based on the parallel primers that target several unspecified genomic sequences. RAPD has been widely used for the typing of bacterial isolates in cases of disease outbreaks (Lenin *et al.,* 2011). Poultry provides income for small and medium scale farmers in Nigeria, but suffers from major limitations of disease like *Salmonella* which results in low egg production, low performing breeds and poor feed conversion. This study is necessitated due to the increasing complain of poultry farmers of incidences of *Salmonella* in the study area.

Materials and Methods

Study Area

This study was conducted in Abia State Southeast of Nigeria. Abia State lies between latitude $4^{O} 40^{1}$ and $6^{o} 14^{1}$ north and longitude $7^{o} 10^{1}$ and $8^{o} 10^{1}$ east. The state is bounded by Rivers State in the south, Ebonyi State in the north, Imo State in the west and Akwa Ibom State in the east. The population of Abia State is 4.3 million, while farming and trading are the major occupation (FRN, 2007).

Study Population

Chickens in poultry farms and hatcheries in Abia State contributed the study population.

Sampling Technique and Sample Collection

A multistage sampling method was employed in this study. In the first stage, Abia State was purposively selected out of

the 5 states in the region based on complaint by many farmers of failures of fowl typhoid vaccine administered to their birds. The sample collection cut across the three senatorial zones that make up the state. In the second stage, two Local Government Areas (Aba south and north, Ikwuano and Umuahia south, Ohafia and Bendel Local Government Areas of Abia State) were randomly selected from each of the three senatorial zones in the state.

Egg, cloacal swabs and litter samples were used for the study. Eggs were collected and placed in polythene and then kept in a plastic box and transported to the veterinary laboratory for processing. Cloacal samples were collected by placing sterile swab stick into the vents of the chicken, gently rotated before withdrawal while the litter was collected by using sterile spatula to collect 10g of litter on the floor of the poultry house into a sterile universal bottle and then transported to the laboratory for processing. The sample collection lasted for a period of 10 months, from July 2014 to April 2015.

Isolation of *Salmonella* from Egg, Cloacal and Litter

Isolation of *Salmonella* was done according to the procedure described by Zancan *et al*. (2000). A loopful of pooled egg were inoculated into peptone water (enrichment broth) and incubated at 37°C for 24hour. Each cloacal swab sample was inoculated into peptone water (Pre-enrichment broth) and incubated at 37oC for 24 hours. Similarly, for poultry litter approximately 5g of poultry house manure were placed into a universal bottle containing 10ml of physiological buffer saline. The poultry manure samples were inoculated into peptone water and incubated at 37oC for 24 hours. All other procedures apply for eggs, cloacal and litter samples. A loopful of the pre-enrichment broth was inoculated into Rappaport-Vassiliads (RV) broth (enrichment broth) and incubated at 42°C for 24 hours. After incubation, the RV broth was streaked on Macconkey agar and deoxysocholate citrate agar. Inoculated plated were incubated overnight at 37°C and observed for colourless (non lactose fermenting) colonies suspicious of *Salmonella*. The *Salmonella* suspected colonies were subcultured to MacConkey media for purification.

Biochemical Tests

Biochemical tests were conducted using Simmon citrate agar, Urease agar, Triple sugar Iron, Sugar fermentation test Motility test, Methyl red and Vogues Proskauer test to confirm if the isolates were *Salmonella* phenotypic ally.

Genomic DNA Extraction Protocol

Genomic DNA was extracted using the boiling method according to the protocol of Danifor Biotechnology (2012) stock culture of the organism was sub-cultured on MCA and incubated at 37°C for 24 hours. After incubation, three colonies of each isolate were collected and added to 200µl buffer AL (containing lysostaphin) and mixed thoroughly by vortexing.

The suspension was incubated at 56°C for 10 mins, after which 200µl absolute ethanol was added, mixed thoroughly by vortexing and then one milliliter of the mixture was pipette into the DNase mini spin column. The spin column was placed in a 2ml collecting tube and centrifuged at ≥6000Xg for 1min. The flow-through and the collecting tube were discarded. The spin column was placed into a new 2ml collecting tube and 0.5ml buffer AWI, was added and centrifuged for 1 min at ≥6000Xg. The flow through and the collecting tube were discarded again. The

spin column was placed in a new tube and o.4ml of buffer AW2 was added and centrifuged at 20,000Xg for 3 mins. The flow-through and the collecting tube were again discarded. The spin column was transferred into a new 1.5 or 2ml micro-centrifuge tube. The DNA was eluted by adding 0.2ml of buffer AE to the center of the spin column and finally centrifuge for 1 min at ≥6000Xg in other to increase the yield.

Amplification of Target DNA

The target DNA was amplified by the Polymerase chain reaction (PCR). The procedure described by Promega Cooperation, Madision USA was conducted in a volume of 25µL containing 20µL of genomic DNA from each *Salmonella* Isolate. A volume of 30µL of the supernatant was used as template for amplification by PCR assay. The sequence of a pair of primer specific for the genus *Salmonella* (16SrDNA341F5-CCT-ACG-GGA-GGC-AGC-AG-3 and 907R5-CCG-TCA-ATT-CCT-TTR-AGT-TT-3), Inqaba Biotechnical Industries South Africa was used.

A known *Salmonella* strain was used as positive control. Reactions with the primer were carried out in a total volume of 25µL amplification mixture consisting of 2.5µL of 10X reaction buffer (500mM KCL, 200mM Tris-HCl) of each primer (10mM), 0.6µL of Taq DNA polymerase (fermentase). 3µL of extracted DNA as template and 9.6L of distilled water. Amplification was performed in Techne TC512 thermocycle. The cycling conditions were as follows: 35 cycles of denaturation at 94°C for 30s, annealing at 56°C for 90s, elongation at 72°C for 30s, and final extension period for 10min at 72°C. Amplified products were electrophorased in 1.5% agarose gel and a 100-b DNA ladder was issued as a size maker. After staining with ethidium bromide, the gel were visualized and photo graphed under transilluminator ultra-violet (UV) light with gel documentation apparatus (MB Fermentase USA).

Results and Discussion

A total of 1420 samples obtained from 43 farms in Abia State were processed for *Salmonella* isolation. Out of the 1420 samples processed, 40 (2.81%) produce non-lactose fermenting colonies on MacConkey while only 24 (60.0%) were urease negative. The inability of the isolate to hydrolyze urea and the negative indole-reaction obtained is in agreement with Muktaruzzaman *et al*. (2010). The utilization of Simmon Citrate by the isolates was consistent with the finding of Lee *et al*. (2003).

Table.1 Number of Farms and Local Government Areas Selected For the Study

State	Local government area	No. Of farms selected
Abia	Aba north	14
	Aba south	11
	Umuahia south	6
	Ikwuano	5
	Ohafia	3
	Bendel	4
Total	**6**	**43**

Table.2 Salmonella distribution location and number sampled from eggs, fecal swab and poultry litter in Abia State.

Location	No. sampled	Egg	Cloacal swab	Litter poultry	Isolation rate (%)
Ohafia	60	-	-	-	0.00
Bendel	60	-	1	-	1.66
Umuahia South	180	2	1	-	1.66
Ikwuano	120	1	0	-	0.83
Aba south	610	9	3	2	2.30
Aba north	390	6	2	1	2.30
Total	**1420**	**18**	**7**	**3**	**1.97**

Table.3 Isolation Rate of Salmonella in Poultry Farms in Abia State.

Senatorial zone	No of samples processed	No positive for *salmonella isolates*	Isolation rate (%)
Abia North	200	2	1.00
Abia Central	420	6	1.43
Abia South	800	20	2.50
Total	**1420**	**28**	**1.97**

Fig.1 Map of Abia State showing the three senatorial zones

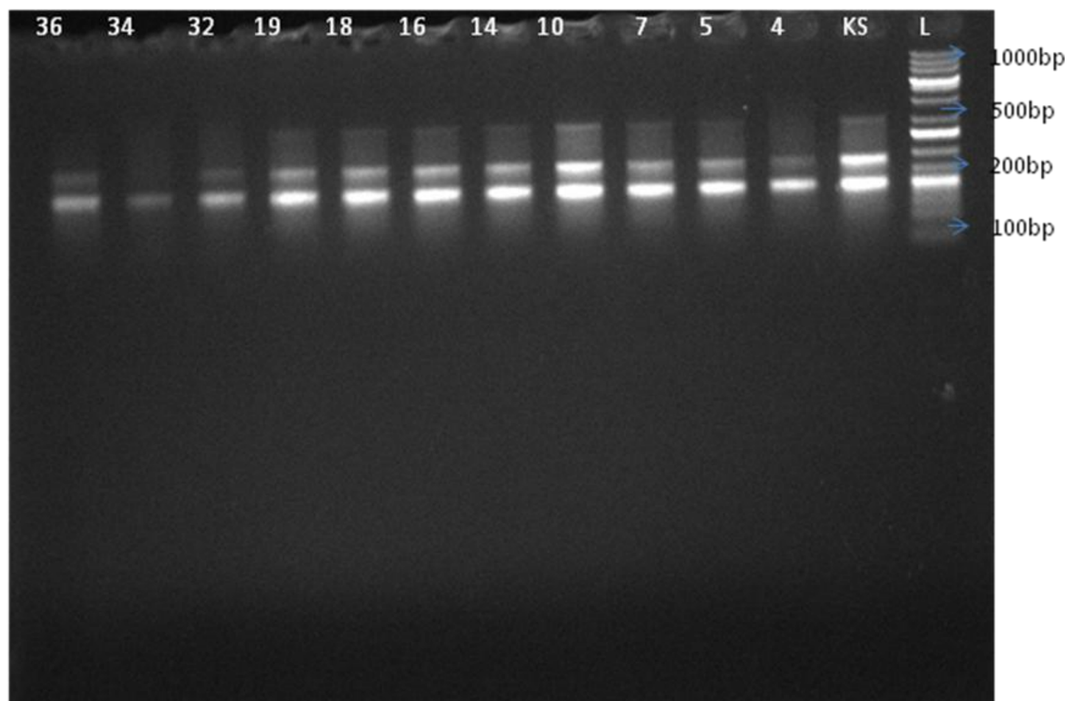

Plate 1: Representative agarose gel of amplified products using universal Salmonella primer set. L is 100bp-1kbDNA ladder (molecular marker). Lanes 36, 32,18,19, 16, 14, 10, 7, 5, 4, are positive for *Salmonella species* with bands at 200 while lane 34 is also positive for *Salmonella species* with band at 200bp.KS is known *Salmonella* strain

The 24 isolates agglutinated in *Salmonella* poly O antiserum but did not agglutinate poly H. The distribution of Salmonella in the six local government areas ranges from 0.0% to 203%.

The study shows that Aba north and south has the highest with isolation rate of 2.30% each while Ohafia local government area has the least with 0.0% suggesting that farms in Ohafia local government area are Salmonella free. This may be due to high management levels practiced by the poultry farmers in that area and right use of vaccine. The isolation rate ranges from 1.00 – 2.50% in the three senatorial zones of Abia State with Abia south having the highest predominant isolation rate of 2.50%. There was no significant association (P > 0.05) between isolation rate and senatorial zone.

From the study, the *Salmonella* isolation rate in Abia State was 1.97% and this is closely related to the findings of Suresh *et al.* (2006) who reported a 1.86% isolation rate in new Delhi, India but in disagreement with Al-Abadi *et al.* (2011), who reported *Salmonella* isolation rate of 9.2% in Dharka, region of Bangladesh. The reason for this low isolation rate may be due to several factors like use of vaccination, good hygiene practices, restriction of poultry farm attendant from moving from one poultry farm to the other and the source of day old chicks.

Universal primer set were used to detect/confirm the *Salmonella* species by Polymerase chain reaction. Salmonella species processed by PCR produced bands with amplicon size of 200bp following gel electrophoresis and ethidium bromide staining of the PCR product (Plate 1).

This is in line with the work carried out by Dione *et al.* (2011), but in disagreement with the findings of Zahravi *et al.* (2005). Zahravi *et al.* (2005) in his work carried out in\ Shiraz City in South of Iran using different primers reported amplification of *Salmonella* DNA with amplification size of 284bp. The difference in amplification size could be due to differences in primer type use. This finding is closely related to that of Kwon *et al.* (2010) who reported the amplification of *Salmonella* with amplicon size of 197bp. The primer type used in this study is the same with that used by Kwon *et al.* (2010).

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