Salmonella Biovars Isolated From Poultry Farms in Abia And Imo States South Eastern Nigeria

'Nwiyi, P. O, [2]Chah, K. F and[3]Shoyinka, V. O.

'Department of Veterinary Microbiology, College of Veterinary Medicine, Michael Okpara University of Agricuture, Umudike.

[2,3]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria Nsukka.

Abstract: The aim of this study was to evaluate the biovars of *Salmonella* that are found in poultry farms in Abia and Imo States, Southeastern Nigeria. Forty *Salmonella* isolates obtained from previous studies were subjected to molecular identification using the polymerase chain reaction (PCR) with *Salmonella* universal and species specific primers. Six different *Salmonella* serotypes were screened to determine the biovars that was present in the study. The serotypes was *Salmonella infantis, Salmonella typhimurum. Salmonella weltervreden. Salmonella enteritidis. Salmonella pullorum* and *Salmonella gallinerum.* The PCR products of the genomic DNA extracted from the 40 *Salmonella* isolates produced bands at 250bp following agarose gel electrophoresis. Amplification of the species specific primers were evidenced with detection of bands. Two Salmonella biovars were detected out of the 6 Salmonella serotypes used for the study, namely, *Salmonella pullorum* and *Salmonella gallinerum.* Sixteen *Salmonella gallinerum* were isolated from Imo State while 24 *Salmonella pullorum* were isolated from Abia State with bands ranging from lOObp to 250bp. The study shows that the predominant biovars present in the study area were two and this could have remarkable epidemiological implications in the control of the disease. There is need to monitor the movement of day old chicks for pathogenic *Salmonella* species to facilitate the control of these economically important zoonotic *salmonella* disease.

Keywords: Abia State, bands, Imo State, polymerase chain reaction, *Salmonella* biovars.

Introduction

Salmonellae are Gram-negative, short plump rods, non-spore forming, non-capsulated, aerobic and facultative anaerobic organisms and classified under the Family Enterobacteriaceae (OIE Manual, 2006). Although over 2300 serotypes of *Salmonella* have been identified, only about 10% of these have been isolated from poultry (Gast, 1997). Based on molecular studies, the Genus *Salmonella* has been classified into two species namely *Salmonella enterica* and *Salmonella bongori* (Reeves *et al.*, 1989). *Salmonella enterica* is divided into six phylogenic groups: enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV) and indica (VI); the more commonly isolated subspecies are arizonae, diarizonae and houtenae species (Centre for Disease Control, 2003) and subspecies I is most responsible for infections in human and domestic animal. On the basis of differences between the O (cell wall) and H (flagella) antigens, the *salmonella* is classified into several serotypes (Luderite *et al.*, 1966: Grimont and Weill, 2007). Lipopolysaccharide is a major cell wall component comprising lipid A which is a core oligosaccharide and O antigenic polysaccharide chain (Luk and Lindberg, 1991).

Avian salmonellosis is principally caused by the two host specific *Salmonella enteric* biovarsviz: *Salmonella enterica pullorum* and *Salmonella enterica gallinarum* (Snoeyenbos, 1991). Pullorum Disease (PD) is usually caused by *Salmonella enterica pullorum* and is usually confined to the early stages of life and occasionally occurs in adults (Shivaprashad, 1997). *Salmonella enterica pullorum* is transmitted vertically via eggs from parents to offspring. Fowl typhoid (FT) caused by *Salmonella enterica gallinarum* is a chronic disease that most often affects mature birds and is a serious problem resulting in mortality and lowered egg production and hatchability (Christensen *et al.*,1997). *Salmonella enterica gallinarum* can produce lesions in chicks indistinguishable from those associated with pullorum. Village chickens can act as a reservoir of salmonellosis (Bouzuobaa *et al.*, 1992). Typical clinical signs of *Salmonella* infection are loss of appetite, drooping wings, weakness, palor, bacillary whitish diarrhea, greenish-yellowish diarrhea and sudden death (Talha *et al.*, 2001). Transmission is primarily through egg but also via direct or indirect contact with infected birds. Infection transmitted via egg or hatchery contamination usually results in death up to 2-3weeks of age (Wigley *et al.*, 2001). Birds that survive clinical disease when infected at a young age may show few signs of infection but can act as carriers (Berchieri *et al.*, 2001).

Salmonellae are zoonotic bacteria and are associated with disease of veterinary importance. The worldwide distribution of human salmonellosis often parallels the patterns of trade of animal products and food and the migration patterns of humans and animals (Callaghan and Simmons, 2001; Wong *et al.*, 2007; Gilbert *et al.*, 2010). Environmental factors such as air, dirty litter, unclean facilities and vectors such as insects, humans and rodents are responsible for Salmonella. Due to the resistance of *Salmonella* to several antibiotics and absence of documented report of the *Salmonella* types responsible, the need to know the

*Corresponding author:
afodiakechukwu@yahoo.com, [1]Nwiyi, P. O,

Nigerian Journal of Microbiology 2018, 32(1): 4262-4268
Published online at www.nsmjournal.org

biovars of *Salmonella* in Abia and Imo Sates, South east Nigeria is therefore justified.

Materials and Methods

Bacterial isolates

Stock cultures of *Salmonella* were used for the study. The isolates were obtained from chickens in each of the three senatorial zones of Abia and Imo States and two local government areas from each of the senatorial zones of both states. Samples collected were egg, cloacae swab and farm litters. The procedures for isolation was as described by (Nwiyi *et al.* 2016)

Molecular Identification

Genomic DNA extraction protocol

Genomic DNA was extracted using the boiling method according to the protocol of Danifor Biotechnology (2012). Stockculture of each test organism was sub-cultured on MacConkey agar and incubated at 37^{O}C for 24hrs. After incubation three colonies of each isolate were collected and added to 200ul buffer AL (containing lysostaphin) and mixed thoroughly by vortexing. The suspension was incubated at 56^{0}C for 10mins, after which 200ul absolute ethanol was added, mixed thoroughly by vortexing and then one milliliter of the mixture was pipetted into the DNase Mini Spin column. The spin column was placed in a 2ml collecting tube and centrifuged at $\geq$ 6000x g 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 AW1, was added and centrifuged for 1min at $\geq$ 6000 x g. The flow through and the collecting tube were discarded again. The spin column was placed in a new tube and 0.4ml of buffer AW2 was added and centrifuged at 20,000 x g for 3mins. 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 membrane and incubated at room temperature for 1min and finally centrifuging for 1min at $\geq$ 6000 x g in other to increase the yield, the final step was repeated.

Amplification of target DNA

The target DNA was amplified by the Polymerase Chain Reaction (PCR). The procedure as described by Promega Corporation, Madison USA was conducted in a volume of 25ul containing 20ul of genomic DNA from each *Salmonella* isolate. A volume of 30ul of the supernatant was used as template for amplification by PCR assay .The sequence of a pair of primer specific for the genus Salmonella (16SrDNA341F 5-CCTACGGGAGGCAGCAG-3 and 907R 5-CCGTCAATTCCTTTRAGTTT-3), Inqaba Biotechnical Industries South Africa, was used. A Known *Salmonella* strain was used as positive control.

Reactions with this primer were carried out in a total volume of 25ul amplification mixture consisting of 2.5ul of 10X reaction buffer (500mM KCl, 200mM Tris-HCl), 0.8ul dNTPs (10mM), 1ul $MgCl_2$ (50mM), 1.25ul of each primer (10mM),0.6ul of Taq DNA polymerase (Fermentase) 3ul of extracted DNA as template and 9.6ul of distilled water. Amplification was performed in Techno TC512 thermocycle. The cycling conditions were as follows: 35 cycles of denaturation at 94^{o}C for 30s, annealing at 56^{o}C for 90s, elongation at 72^{o}C for 30s, and final extension period for 10min at 72^{o}C. Amplified products were electrophoresed in 1.5% agarose gel and a 100-bp DNA ladder was used as a size maker. After staining with ethidium bromide, the gels were visualised and photographed under transilluminator ultraviolet (UV) light with gel documentation apparatus (MB Fermentase USA). DNA from each *Salmonella* positive isolates was subjected to amplification using *Salmonella* species specific primers (Table 2). The species were identified by the presence of bands specific for the primers that were used.

Results

Salmonella biovars isolated from the Poultry farms in Abia and Imo States

Primer sets specific for six *Salmonell a*biovars (*S. Infantis, S. typhimurum, S. weltervreden, S. enteritidis, S. pullorum* and *S. gallinarum*) were used for biovar identification of the *Salmonella* isolates. Twenty four of the 40 *Salmonella* isolates were amplified by *S. pullorum* specific primers as evidence by bands of approximately 250bp following gel electrophoresis and ethidium bromide staining of the PCR products (Plate 2). Sixteen of the 40 isolates were amplified by *S. gallinarum* specific primers and bands of approximately 100bp were produced as shown (Plate 3).

No bands were detected when the PCR products of *S.* Infantis, *S. typhimurum*, *S. weltervreden* and *S. enteritidis* were viewed under Ultraviolet Fermentase Apparatus (UFA-USA). Thus, 24 (60.0%) of the 40 isolates were *S. pullorum* and 16 (40.0%) were *S.* gallinarum (Table II). Fourty percent of the *S. pullorum* biovars were recovered from eggs while 15% of the *S.* gallinarumbiovars were isolated from the cloaca (Table III). Sixty nine point two percent (69.2%)of the *S. pullorum* biovars were recovered from Abia State, while 30.8% of the *S. gallinarum* biovars were isolated from Imo State (Table IV).

Table I: Primers used for *Salmonella* confirmation and identification

Name of Primers	Primer sequence
16SrRNA341F (Sal. Sp.)	CCTACGGGAGGCAGCAG
907R (Sal. Sp.)	CCGTCAATTCCTTTRAGTTT
S. pullorum. F	CTGGTGATGACGGTAATGGT
S. pullorum R	CAGAAAGTTTCGCACTCTCG
PuTKanaR1(S. gallinarum)	GCGGCCTCGAGCAAGACGTTT
Tag 1 (S. gallinarum)	GTACCGCGCTTAAACGTTCAG
SefB127L (S. enteritidis)	AGATTGGGCACTACACGTGT
SefB661R (S. enteritidis)	TGTACTCCACCAGGTAATTG
S. weltervreden F	GATCATCCATTCGGCATTAAACA
S. weltervreden R	CTAAATGGGAAGGCAGCGACTC
558F (S. infantis)	AACAACGACAGCTTATGCCG
1275R (S. infantis)	CCACCTGCGCCAACGCT
A30bpF (S. typhimurium)	CGGAACGTTATTTGCGCCATGCTGACCT AG
A27bpR (S. typhimuruim)	GCATGGATCCCCGCCGGCGAGATTGTG

F = forward, R=Reverse

Table II: *Salmonella* biovars detected in poultry farms in Abia and Imo States

Salmonella serotypes	No detected	% detected
S. infantis	0	0.0
S. typhimurum	0	0.0
S. weltervreden	0	0.0
S. enteritidis	0	0.0
S. pullorum	24	1.19
S. gallinarum	16	0.79
40	1.98	

Table III: Distribution of *Salmonella* biovars based on sample type

		No (%)* of *Salmonella* biovar			
Salmonella biovars	Total No	Egg	Cloaca	Farm(litter)	Total
S. pullorum	24	16 (40%)	6 (15%)	2 (5%)	24(60%)
S. gallinarium	16	8 ((20%)	6 (15%)	2 (5%)	16(40%)
Total	**40**	**24 (60%)**	**12 (30%)**	**4(10%)**	**40(100)**

*=% of total number of *Salmonella* isolates.

Table IV: Prevalence of *Salmonella* biovars isolated from poultry farms in Abia and Imo State

	No (%)* of *Salmonella* biovar	
Biovars	Abia	Imo
S. pullorum	18 (69.2)	6(42.9)
S. gallinarium	8 (30.8)	8(57.1)
Total	**26(100%)**	**14(100)**

□ = % of total number of *Salmonella* isolates

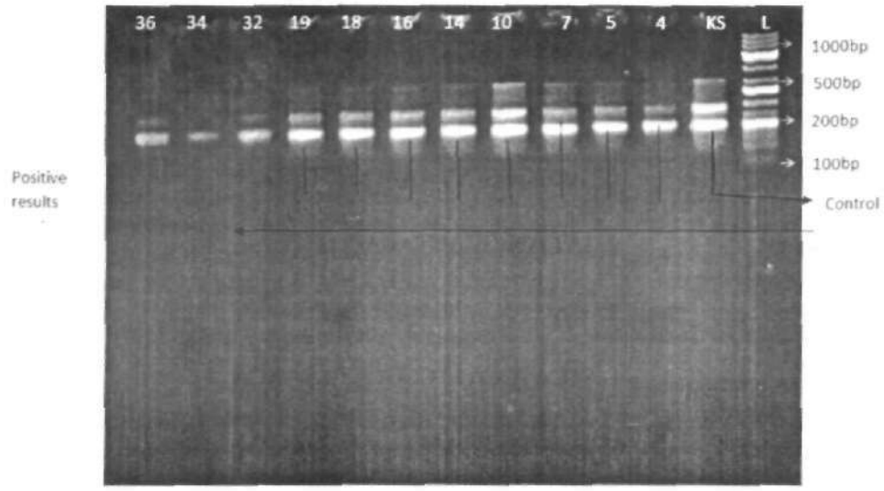

Plate 1: Representative agarose gel of amplified products using universal *Salmonella* primer set. L is 100bp-1kbDNA ladder (molecular marker). Lanes 36, 32,18,19, 16, 14, 10, 7, 5, 4, are positive for *Salmonella species* with bands at 200 while lane 34 is also positive for *Salmonella species* with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

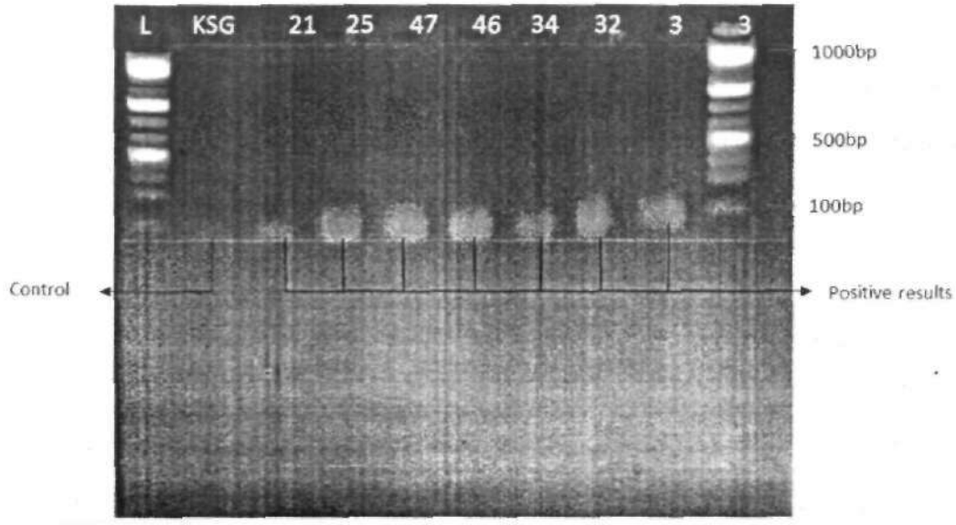

Plate 2: Amplified products of bacteria isolates analyzed with 1.5% agarose gel electrophoresis. L is 100bp-1kb DNA ladder (molecular marker). Lanes 21, 25, 47, 46, 34, 32 and 3 are positive for *Salmonella gallinarum* with bands at 100bp.KSG is known *Salmonella gallinarum*

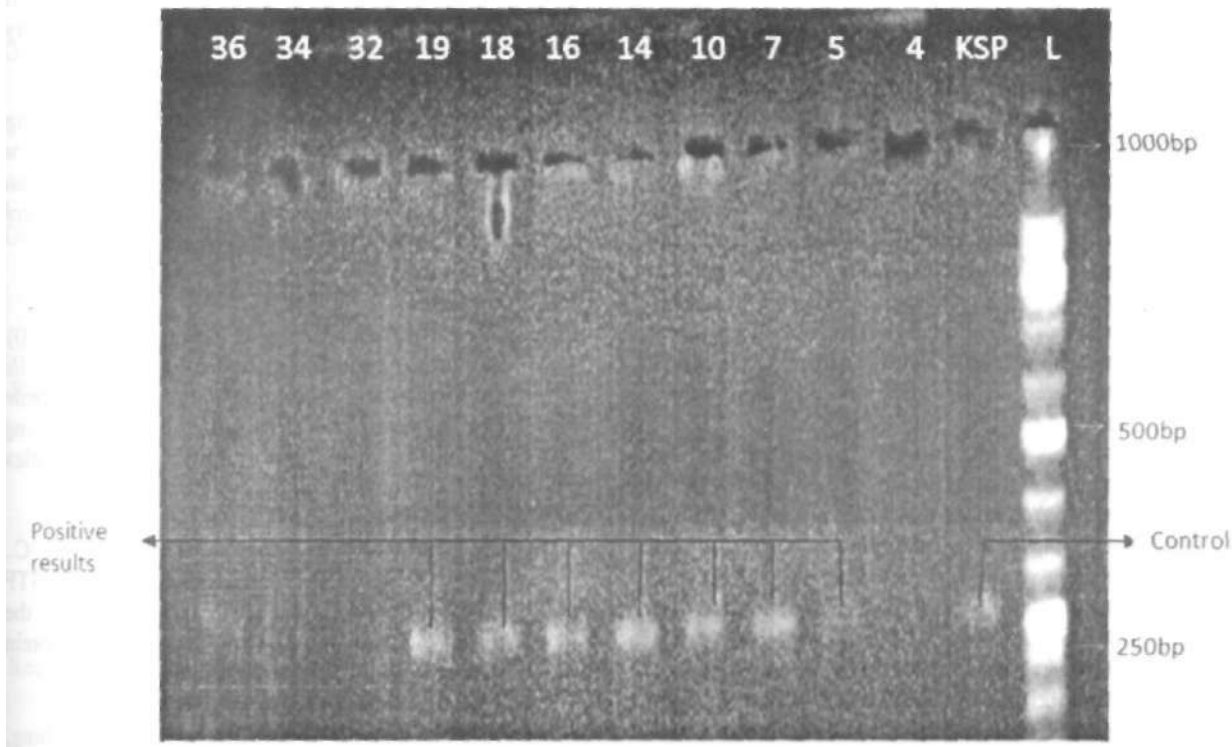

Plate.3: Representative agarose gel of amplified products using specific primer set L is 100bp-1kb DNA ladder (molecular marker). Lanes36, 19, 18, 16, 14, 10, 7, and 5 are positive for *Salmonella pullorum* with bands at 250bp whereas lanes 34, 32 and 4 are negative for *SalmonellaPullorum*.KSP is known *Salmonella pullorum*

Discussion

In the study, more *Salmonella pullorum* was isolated in Abia state than Imo state, this may be due to a number of factors among which includes the source of the birds. Since Abia state does not have a hatchery, they depend on the hatchery from western part of Nigeria particularly Oyo state for their chicks and incidence of *Salmonella pullorum* had been reported from there(Saba *et al.* 2011).Most birds in farms in Imo state comes from the hatchery in located in that state of which pullorum outbreak had not been reported. Also, the level of sanitation and bio-security observed in farms in Abia state may be poorer compare to those in Imo state. The famers in Abia state possibly visit the laboratory less with samples for diagnosis than farmers in Imo state. Since the abuse of antibiotics constitutes a problem of resistance by implication, the farmers in Abia state tend to seek the intervention of veterinary doctors less than those of Imo state. The isolation of these *Salmonella* biovars present a risk to other South east states due to the proximity to source of infection and this may also impact negatively to Nigeria at large due to movement of eggs from one part of the country to another for sell to the end users. The *Salmonella* isolates in this study belonged to the same serogroup and because of the difficulties in identifying them, the DNA of the 40 *Salmonella* isolates were amplified by primers specific for this *Salmonella* biovars, namely *S. gallinarum* and *S. pullorum* producing sizes of 100bp and 250bp, respectively. The amplicon size for *S. gallinarum* is similar to the 97bp amplicon size reported by Stegniy *et al.* (2014), and the primer set used for this study was the same as that of Stegniy *et al.* (2014) but different from those of Narges *et al.* (2014) and Kwon *et al.* (2010). However, the amplicon size for *S. gallinrum* in this study is different from the 200bp and 197bp recorded by Narges *et al.* (2014) and Kwon *et al.* (2010), respectively. The primer set used in this study is different from that use by Kwon *et al.* (2010). In the present study, *Salmonella pullorum* were amplified with primer set to produce an amplicon size of 250bp and differs from the 197bp obtained by Kwon *et al.* (2010). Oslen *et al.* (1996), analyze *S. pullorum* and *S. gallinarum* strains using molecular typing method and reported their extreme similarity in terms of chromosome constitution. Though these *Salmonella*

biovars are very similar in relation to their antigenic properties, dulcitol fermentation has been used to differentiate them. The differentiation between these two *Salmonella* is very important both from an epidemiological standpoint and in relation to control programs, sanitary measures to be adopted would be different Sambrook (2001). Robinson *et al.* (2001) and Rahman, (2003), reported dulcitol fermentation test as a test performed to differentiate non-motile *S. pullorum* from *S. gallinarum* worldwide.

The two *Salmonella* biovars isolated in this study are responsible for avian salmonellosis. *S. gallinarum*is the causative agent of fowl typhoid which affects more of adult birds, has been responsible for high mortality and poor egg production. While *S. pullorum* is the cause of *pullorum* disease which affects more of young chickens under 4weeks of age and has been responsible for high mortality in Nigeria and other countries of the world. Avian salmonellosis may occur in chickens when subjected to undue stress, hence, birds should be vaccinated using both live and inactivated vaccines.

In this study, *Salmonella pullorum* was the predominant biovar isolated from egg contents. This observation is in agreement with Oliveira *et al.* (2003) who reported *S. pullorum*as the predominant biovars isolated from egg contents in Sao Paulo, Brazil. However, Olorunsola *et al.* (2012) found *S. gallinarum* as the most frequently isolated *salmonella* biovar in eggs in hatcheries in south west Nigeria while Jebelli*et al.* (2012) reported that *S. enteritidis* was the most frequently isolated from egg contents in Semnan, Iran. Musgrove *et al.* (2005) and Jamshidi (2010) found *Salmonella enteritidis* and *S. typhimurium*, respectively to be the most frequently isolated serovars from egg content. These variations may be a reflection of the predominant biovars in the study area.

Conclusion

Salmonella used for this work were isolated from egg, cloacae swab and farm litters (environmental samples). Of all the *Salmonella* isolates, only 2 biovars namely: *salmonella gallinarum* and *salmonella pullorum* were isolated. This shows that within the area of study, and indeed in southeastern Nigeria, these are the predominant biovars.

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