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Full Length Research Paper

Identification of Pathogenic Genes in Salmonella Strains from Poultry and Associated Wastewater

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The study was conducted in Aba, the commercial city of Abia State to determine the prevalence of Salmonella on chicken and chicken water waste using fecal samples from apparently healthy birds, clinically sick birds and swabs of waste water effluent from chicken farms. A total of 180 samples made of 60 samples each were collected from the three sample types. Biochemical and Serological analysis were conducted in veterinary laboratory of Michael Okpara University of Agriculture, while molecular studies were conducted at Lahor Medical Research and Diagnostic Center, Edo State Benin. The prevalence of *Salmonella Spp.* in apparently healthy chicken, clinically sick chicken and chicken water waste were 6.6% 11.6% and 20% respectively, while the mean prevalence rate was 12.7%. Polymerase chain reaction (PCR) was used for detection of virulence genes with known amplification conditions, number of cycles and concentration of reagents while amplicon were separated by electrophoresis in a 1.5% agarose gel. The detection of V_i and V_3 genes with bands at 100bp and 150bp suggest the presence of virulence genes of *Salmonella Typhimurium,* while the detection of *inVA* and *sopE* genes with bands at 260bp and 240bp indicates the presence of virulence genes for *Salmonella Enteritidis.*

Keyswords: Salmonella, chicken samples, prevalence, virulence genes, amplification conditions.

INTRODUCTION

Salmonella are short bacilli, Gram-negative, aerobic, catalase positive, oxidase negative; they ferment sugars with gas production, produces H_2S, are non sporogenic and are normally motile with peritricheal flagella, except for *Salmonella Pullorum* and *Salmonella Gallinerum*, which are non-motile (Plym and Wierup, 2006). These organisms are Gram negative and rod shape which have been divided into over 2700 serotypes based on somatic, flagellar and capsular antigens (Gallegos-Robles *et al.*, 2003). The genus salmonella is divided into two species *Salmonella*

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enterica and *Salmonella bongari.* They are 6 subspecies of *Salmonella enterica* and they include *S.enterica subsp arizonae S. enterica subsp diarizonae, S. enterica subsp-enterica*, *S. enterica subsp indica*, *S.enterica subsp houtenae or I, II IIIa, IIIb, IV* and *VI.* (Hendriksen *et al*., 2011). Salmonella Spp have contributed significantly to the cause of foodborne disease in humans and the most frequently isolated from Asia, South America and Europe (Vieira *et al.*, 2009). Analysis of the phylogenetic characteristics unfold the influence of varying factors in the existence and continued presence of Salmonella Spp in the animals and these include; Gross-contamination among animals, feed and environment (Mello *et al* 2011. et al). Salmonella Spp virulence had been traced to two major factors notably chromosomal and plasmid mediated factors (Oliveria, *et al*., 2003). Studies have shown that genes encodes some factors and these virulence factors are associated with the fimbriae and celluar structure (Edwards and Puente, 1998: Van Asten and Van Dijk, 2005). Other virulence factors of salmonella include production (of endotoxins and exotoxins. Salmonella encoded frumbria (*sef* operon) functions to promote a better interaction between the bacteria and the macrophages (Collinson *et al.,* 1996). Bacteria affinity for payer's patches and adhesion to intestinal M cells is associated with the long polar fimbria (Baulmer *et al.*, 1996). Most virulence genes of salmonella are chromosomal genes located on the pathogenicity islands called Salmonella Pathogenicity Islands (SPI). These genes are thought to have been acquired from other bacteria species via gene transfer (Ezzat *et al.*, 2014).

The study was carried out due to several complain by farmers of outbreak of Salmonella in the area and hence the study was aimed to detect the presence of virulence genes in salmonella isolated from chickens and chicken waste water.

MATERIAL AND METHODS

Sample Collection

A total of 180 samples obtain from chicken poultry and poultry waste water was collected for isolation of salmonella. These samples were made of fecal swabs of healthy birds, suspected weak birds and swabs of waste water effluent from poultry farms. All samples collected were kept in sterile plastic bags containing ice pack and then transported to veterinary microbiology laboratory of Michael Okpara University of Agriculture, Umudike.

Isolation of Salmonella

The procedure as described OIE (2004) was employed. The samples from each of the three classes was separately inoculated into peptone water and incubated at 37% for 24hrs. Thereafter a loopful of the enriched media was stroked onto plates of *Salmonella – Shigella* agar and deoxycholate citrate agar and incubated at 37^{O}C for 18-24hrs. The characteristics colony was picked for further studies.

Identification of *Salmonella* Isolates.

Microscopic, biochemical and serological identification were further carried out. The purified colonies were subjected to Gram's reaction and on microscopy, they were colourless, transparent and raised colonies on *Salmonella-Shigella* agar but smooth, red coloured colony with black central spot in deoxycholate citrate agar. The biochemical tests were oxidase, indole test, methyl red, Vogues – Proskauer, citrate utilization, Tripe sugar iron agar test (TSI) and sugar fermentation test using analytical profile index 20E (API) while serology was carried out by slide agglutination test using O and H antisera (Difco, Detroit, USA)

DNA Extraction

The stock frozen bacteria culture was sub-cultured in MacConkey agar plates (Oxoid, United Kingdom) and incubated at 37^{O}C for 24hr. An alliquot (ImL) of each bacterial culture was separated for DNA extraction according to the method described Borsol *et al*. (2009). Polymerase chain reaction (PCR) specific primers for the following genes: *Vi*, *V_3, inVA* and *sopE* were used for conduction of polymerase chain reactions. The primer sets, products and source used in the assay of PCR are described (Table 1). The PCR mixtures were performed with 2.5 $\propto$L of 10xPCR buffer (Inqaba biotechnology industry Limited, South Africa), I U of Taq DNA polymerase and 2$\propto$L of template DNA. The amplification conditions, number of cycles and concentration of reagent are described (Table 2). The amplicons were separated by electrophoresis in a 1.5% agarose, stained with ethidium bromide and viewed using UV documented transilluminator. *Esherichia Coli ATCC 25922* was used as negative control. All the procedures were conducted in Lahor Medical Research and Diagnostic Center, Benin, Edo State (Nigeria).

DISCUSSION

Salmonella infection is one of the most important bacterial diseases in poultry causing heavy economic loss through mortality and reduced production. In the present study, the incidence of salmonella in chickens was 12.7% out of 180 chickens and chicken waste water samples. This finding is similar to the result obtained (Kudaka, *et al.,* 2006) but disagrees with Fofana *et al.,* (2006) who reported salmonella present in similar study at prevalence rate of

Table 1: Virulence genes and factors detected in salmonella isolated from poultry and poultry water waste

Genes	Virulent factors		Primer sequence (5^1-3)	Source
inVA	Invasion	(F)	GTGAAATTACGCCACGTTCGGCAA	Inqaba South /Africa
		(R)	TCATCGCACCGTCAAAGGACAA	
sopE	Effect or protein	(F)	ACACACTTTCACCGAGGAAGCG	Inqaba South /Africa
		(R)	GGATGCCTTCTGATGTTGACTGG	
VI	Fimbria	(F)	CGACTGAAACCGTTGGTACA	Inqaba South /Africa
		(R)	CAATGATCGCAGCGTAGTGG	
V3	Fimbria	(F)	CCAGACTCCTACGGGAGGCAG	Inqaba South /Africa
		(R)	CGTATTACCGAGGCTGCTG	

Table 2: Virulence associated genes in salmonella isolates and its PCR assay conditions.

gene	Amplification condition	Number of cycles	Concentration of reagents
Vi	94^{O}C 1sec, 58^{O}C 1sec, 74^{O}C 21sec.	35	2∝L dNTPs (2.5mM)+each primer+1.25∝L $MgCl_2$ (2.5mM).
V3	94^{O}C 1sec, 55^{O}C 1sec. 74^{O}C 21sec	35	0.2∝L d NTPs (2.5mM)+1uL each primer+2∝L$MgCl_2$ (4mM)
invA	94^{O}C1sec, 56^{O}C1sec, 74^{O}C 21sec.	35	2∝L dNTPs (2.5mM)+1uL each primer+1.25∝L$MgCl_2$ (2.5mM)
sopE	94^{O}C1min, 55^{O}C1min, 72^{O}C1min.	30	2∝L dNTPs (2.5mM)+1uLeach primer+1∝L$MgCl_2$(2mM)

Min=minutes, sec=seconds

RESULT

Two salmonella serotypes (*S. Enteritidis* and *S. Typhimurium*) were examined for detection of virulence genes are *Vi, V_3, inVA* and *sopE* by conventional PCR and the result obtained are presented.

Table 3: The prevalence of salmonella Spp in chicken and chicken waste water.

Examine chicken samples	Number of sample examined	Number of sample positive	%
Apparently healthy chicken	60	4	6.6%
Clinically sick chicken	60	7	11.6%
Chicken waste water	60	12	20%
Total	180	23	12.7%

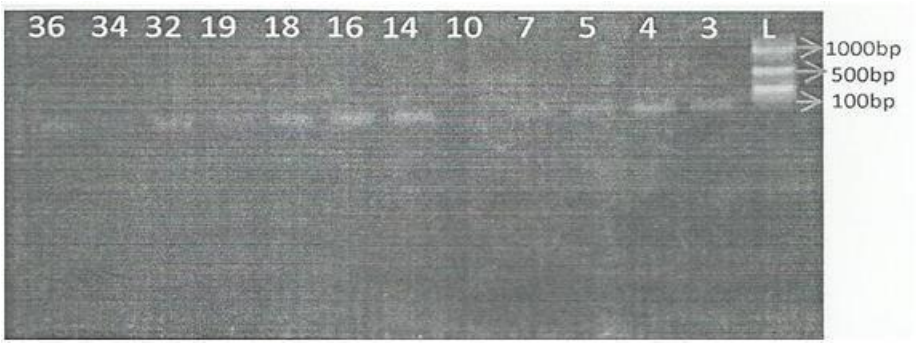

Plate 1: PCR results for *Salmonella* species analyzed with 1.5% agarose gel electrophoresis. L is 100bp-1kb DNA ladder (molecular marker), lanes 3, 5, 7, 14, 16, 18, 19 and 36 are positive for the *Vi* genes of *Salmonella Typhimurium,* isolates 4, 32 and 34 are positive for the *Vi* genes of *Salmonella Typhimurium* at 100bp and isolate in lane 10 is negative for the *Vi* genes.

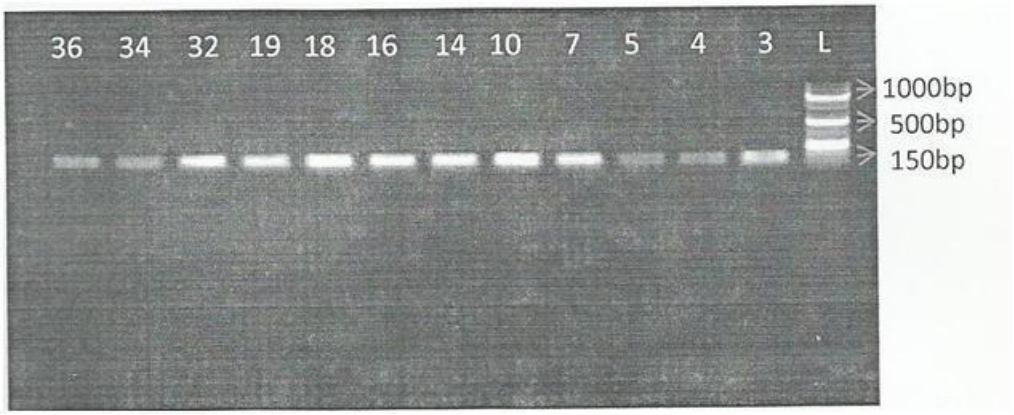

Plate 2: PCR results for *Salmonella* species analyzed with 1.5% agarose gel electrophoresis. L is 100bp-1kb DNA ladder (molecular marker), lanes 3, 5, 7, 14, 16, 18, 19 and 36 are positive for the *V3* genes of *Samonella typhimurium,* isolates 4, 32 and 34 are positive for the *V3* genes of *Salmonella typhimurium* at 150bp respectively.

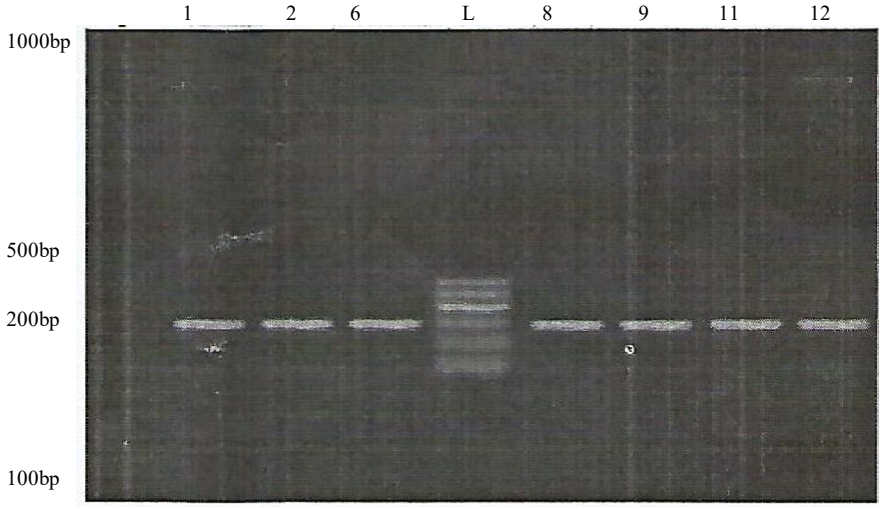

Plate 3: Agarose gel electrophoresis of ampilified *invA* PCR product (240bp). Lane L: 100-1000bp DNA ladder; Neg. Negative control; Lane 1, 2, 6, 8, 9, 11, 12 examined *Salmonella*.

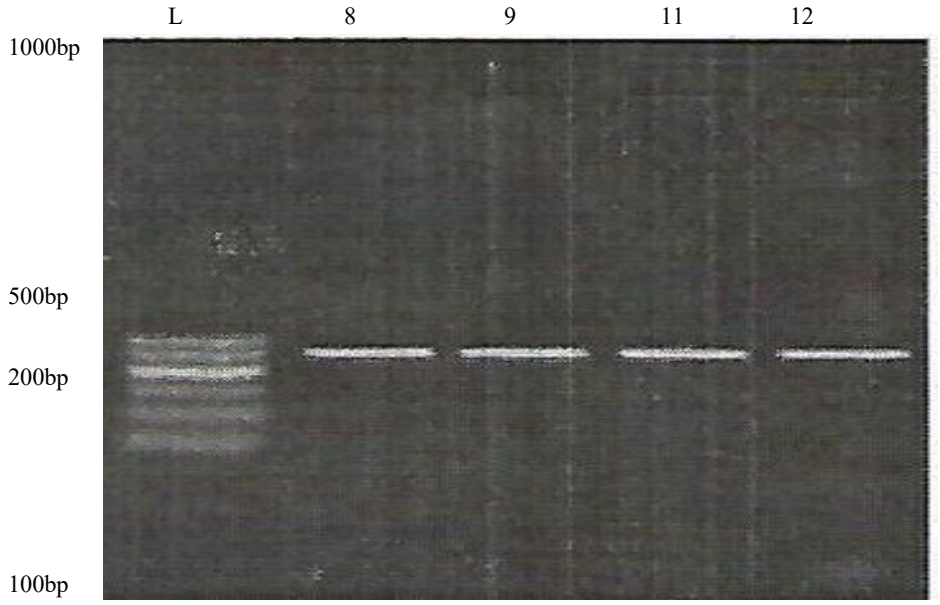

Plate 4: Agarose gel electrophoresis showing *Salmonella* specific PCR of *Salmonella isolates using primer set for the sopB gene (260bp). Lane L: 100-1000bp DNA ladder; Neg: negative control; Lane 8, 9, 110, 12 examined Salmonella*.

62.5%. The reason for this remarkable or significance differences may not be in connected with difference in management, environmental and socio-economic related factors.

The result of serotyping of isolated salmonella specie using "O" and "H" antisera serotypes recovered 12 *Salmonella Typhimurium* (52.2%) and II *Salmonella Enteritidis* (47.8%)

Oliveira *et al.* (2003) revealed that PCR method has high specificity and sensitivity and more importantly a less time consuming procedure than standard microbiological techniques for detection and identification of salmonella. PCR assays using the *inVA* primers specific for *Salmonella spp*. considerably decreases the number of false-negative. The same goes for all other virulence genes used in this work. In this study, salmonella isolate obtained from chicken were positive for *inVA* and *sopE* genes. Similar observation was reported (Campioni *et al.,* 2012; Craciunas *et al.*, 2012). The detection of these genes was not out of place because there are necessary for cell division. PCR is a confirmed tool for rapid detection of Salmonella spp and *sopE* and *inVA* are known to be target genes for the detection of salmonella. There was amplification of the virulence genes *inVA* and *sopE* with amplicon size of 240bp (Plate 3) and 260bp (Plate 4) respectively. The results for detection of *sopE* and *inVA* genes showed 100% positive (frequency) and this is in agreement (Hopkins and Threlfull, 2004). This finding are very significant because variation in components of the proteins and fimbria can lead to a change in the ability of this serovar to adapt to a new hosts thereby resulting in the outbreak of a more virulent strains and this is in agreement with the findings (Prager *et al.,* 2000). However, the absence of this *sopE* effect or proteins in some Salmonella isolates indicates that they are not necessary for invasive activity in human host (Suez *et al*., 2013). The V_1 and *V3* virulence genes are also called fimbria genes and the importance of fimbrae cannot be over-emphasised in infection process. The fimbria genes are thought to posses additive effects of adhesions V_1 and V_3 in the collonization of the intestine and systemic virulence (Wagner and Hensel, 2011). The *Vi* and *V3* genes were found to be present and amplify all the salmonella isolates obtained from chicken waste water as evidenced by the various base pair (bp) bands (Plate 1 and 2). All the 12 salmonella isolates showed marked presence of *Vi* and *V3* genes with amplification at 100bp (plate 1) and 150bp (plate 2) respectively. The findings that 12.7% were positive for *Salmonella Typhimurum* strains isolated from chicken waste water is in agreement (Moussa *et al.,* 2003).

There is a possibility that the presence of this gene may be associated with the host from which the sample was isolated (Amin *et al.,* 2010).

PCR assay carried out for the detection of *sopE* and *inVA* from isolated strains revealed that virulence genes was present in all of the isolates (100%) which was demonstrated by the presence of a 260bp and 240bp PCR product respectively

CONCLUSION

The study shows that isolates of salmonella from chicken for virulence genes *inVA* and *sopE* where those of chicken waste water also positive for V_1 and V_3 genes. These genes which are effect or protein and fimbria may be responsible for the invasive and adhesive function of salmonella.

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