NIGERIAN VETERINARY JOURNAL
ISSN 0331-3026
Nig. Vet. J., September 2018 Vol 39 (3): 189 - 193.
https://dx.doi.org/10.4314/nvj.v39i3.1 **ORIGINAL ARTICLE**

Evaluation of *Salmonella* isolates obtained from Poultry Farms in Abia and Imo States for the Presence of Plasmids

Nwiyi, P. [1]; Chah, K. [2] and Shoyinka, S. V. O.[2]

[1.] College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike, Nigeria. [2.]Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria. *Corresponding author: Email: afodiokechukwu@yahoo.com; Tel No:+2348038755556.

SUMMARY
This study was conducted to determine whether there exists the presence of plasmid from *Salmonella* isolated from poultry farms in Abia and Imo states southeast Nigeria. Forty *Salmonella* isolates were used for the study. The alkaline phosphate method of Birnboin and Doly was employed. Three buffers A, B and C made up of different concentrations and volumes with adjusted pH were used. A (400 mM Tris, 200 mM Na Ethylenediaminetetraacetic acid), B (3 M Na, acatic acid) and C (10 mM Tris, 2 M Na Ethylenediaminetetraacetic acid). The test organisms sub-cultured on MacConkey agar (MCA) were processed at various centrifugation rates and time. The suspected pure plasmid deoxyribonucleic acid was mixed with ethidium bromide and loading dye using agarose gel. The set up was viewed under ultraviolet transillumination with gel documentation apparatus. Plasmid cure was conducted with ten multidrug resistant *Salmonella* isolates. Danifor Biotechnology method was used for the plasmid curing experiment. Three curing agents, sodium deodecyl sulphate curing agents (100), sodium deodecyl sulphate (10 g or 10%) and nutrient broth (100 ml) were used. Plasmid DNA was detected from the *Salmonella* isolates evidenced with bands at 100 bp. There was plasmid cure as supported by the zones of inhibition by some of the antibiotics when compared with the original isolates.

Key words: Cure, DNA, multidrug resistant isolates, plasmid, *Salmonella*

INTRODUCTION
Salmonellae are zoonotic bacteria and are associated with diseases 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). Several factors have been reported to be responsible for pathogenicity of *Salmonella* organisms and these include invasion, adhesion serum resistance and fimbriae (Baumler *et al.*, 1998). These virulence factors are often encoded by plasmids and the plasmids are 50 to 90 kb in size and have been called serovar-specific plasmids (Gulig *et al*., 1993). The virulence plasmids of *Salmonella* are important for bacterial multiplication in the reticulo-endothelial system of warm blooded vertebrates

(Guiney *et al.,* 1994). The virulence of *Salmonella* species is associated with combination of chromosomal and plasmid factors (Oliveira *et al.,* 2003).

Plasmids are defined as double stranded, extra-chromosomal genetic elements that replicate independent of the host cell chromosome and are stably inherited (Ou *et al.,* 1990). There are different types of plasmids that have been reported. Naturally occurring plasmids are wild plasmids found naturally in bacteria (Ou, 1993). Recombinant plasmids are altered plasmids introduced into the bacterium for genetic studies (Summers *et al.,* 1993), while cryptic plasmids are those that serve no known functions (Garner *et al.,* 2007). Metabolic plasmids carry some genes that help in cells metabolism while virulence plasmids carry one or several genes that confer virulence properties on the bacteria cell (Manjistha and Stuart, 2011). Conjugative plasmids are those that are able to induce self-transfer (Grohmann, 2003). Some of the known functions coded by plasmids include: antibiotic resistance, heavy metal resistance, virulence, environmental adaptability and persistence, and metabolic functions that allow utilization of different nutrients (Austin, 1984).

Metabolic plasmids are the plasmids that lack genes to initiate self-transfer but do encode the functions needed specifically for transfer of their own DNA (Gerdes *et al.*, 1985). Suicide plasmids are the plasmids which get transformed to another bacterial cell but do not replicate further (Austin, 1984). Resistance (R) plasmids are large conjugative plasmids that carry one or more antibiotic resistance genes (Hayes, 2000). Resistance plasmid can be conjugative or mobilizable. Usually, R plasmids code for their own replication and they are known to have mobile genetic elements. Fertility (F) plasmids are those that have complete gene set to mediate self-transfer by conjugation. Cells possessing it (donor) are termed F+ and those lacking it (recipient) are termed F-. Previous studies have suggested that the presence of Salmonella plasmid may be responsible for the antimicrobial resistance experienced by most poultry farmers. Since the farmers in the area of study have been complaining of antimicrobial failures in the treatment of Salmonella, the study was therefore conducted to determine the presence of resistance plasmids from *Salmonella* isolates from poultry farms in Abia and Imo states southeast, Nigeria.

MATERIALS AND METHODS

Salmonella isolation

Forty *Salmonella* (40) isolates were used.

DNA amplification and molecular identification of *Salmonella* is according to the procedure described by Nwiyi *et al.* (2016).

Plasmid DNA isolation and detection

The alkaline phosphate method of Birnboin and Doly (1979) was used for plasmid DNA isolation. Three buffers and one solution were used for the isolation: buffer A made up of 400 mM Tris, 200 mM Na EDTA, acetic acid with pH adjusted to 8.0; buffer B made of 3 M Na, acetic acid with pH adjusted to pH 5.5 and buffer C made of 10 mM Tris, 2 M Na EDTA, acetic acid with pH adjusted to 8.0; and lysing solution made of 4% SDS and 10 mM Tris.

Each test organism was sub-cultured on MCA and colonies of the organism were re-suspended in 0.2 ml of buffer A and vortexed for one minute, thereafter 0. 4ml of lysing solution was added. The tubes were inverted 20 times at room temperature and then, 0.3 ml ice cold buffer B was added and vortexed and thereafter kept on ice for 30 minutes. The mixture was centrifuged at 3,000 x g for 15 minutes and 0.7ml of chloroform was added to the supernatant and vortexed. After vortexing, the mixture was centrifuged at 3,000 xg for 10 minutes. One milliliter of absolute ethanol was added to 0.5 ml of aqueous layer and kept on ice for 1hour and then centrifuged at 3,000xg for 30 minutes and supernatant discarded. The pellet was washed with 70 % ethanol, decanted and

dried. One hundred microliter or 0.1 ml of buffer C was
added to obtain a pure plasmid DNA. Ten micro liter of 100 bp molecular maker was loaded and mixed with ethidium bromide dye in the first well while 10 ul of each sample + 2 ul loading dye were loaded in the other wells of agarose. The set up was run at 90 v for 60 minutes in a 0.8% agarose gel and viewed under ultraviolet transillumination with gel documentation apparatus (MB Fermentase United State of America).

Plasmid curing

The procedure as described by Danifor Biotechnology (2012) was used. Ten multidrug resistant isolates were used in plasmid curing experiment. Three components make up the material that was used for plasmid curing viz: SDS curing agents (100), SDS (10 g or 10%) and nutrient broth (100 ml). The pH of these three components was adjusted to 7.6 and steamed for 1hour and finally autoclaved again. Half a mililtre of nutrient broth was inoculated with aliquot collected from overnight culture grown in Luria Bertani broth containing antibiotics and incubated for 24 hrs 37 ˚C. Inoculated nutrient broth was incubated for 3-4hrs to allow minimal growth of the organisms. SDS curing agent sufficient to bring the concentration to 1 % (W/V) was added to the nutrient broth, thereafter incubated for 24-48 hrs at 37 ^{o}C. The zone of inhibition of the suspected cured *Salmonella* isolates were recorded and compared with the original isolates.

Data presentation and analysis

Effect of curing on antimicrobial resistance was presented in tables. Presence of plasmid was presented in figures. All analyses were performed at 5% significant level.

RESULT

Plasmid DNA from the 40 *Salmonella* isolates was examined with 0.8%agarose gel electrophoresis. Plasmid DNA was detected in 24 (60.0 %) of the 40 isolates screened. The plasmids were detected at 100 bp (Figures 1 and 2). Out of the 10 *Salmonella* isolates used for plasmid curing, four of the isolates were cured. Gentamycin, levofloxacin and perfloxacin were the antibiotics that exhibited curing. The inhibition zone before treatment with ethidium bromide the plasmid curing agent was 14 mm, 14 mm, 18 mm, 6 mm and 6 mm.

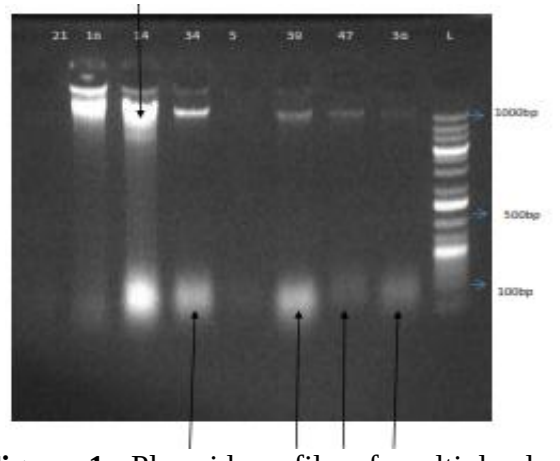

Figure 1: Plasmid profile of multiple drug resistant *Salmonella* species analyzed with 0.8% agarose gel electrophoresis. L is 100 bp-1kb ladder (molecular marker). Lanes 36, 47, 34, 39 and14 are positive for plasmid at 100 bp and Lanes 5and 21 are negative for plasmid

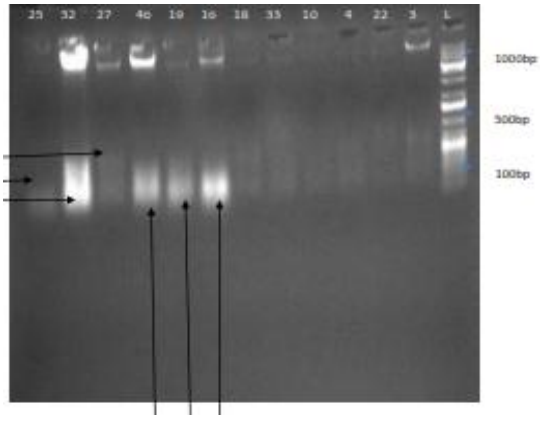

Figure 2: Plasmid profile of multiple drug resistant *Salmonella* species analyzed with 0.8% agarose gel electrophoresis. L is 100 bp-1kbladder (molecular marker). Lanes 25, 32, 27, 46, 19 and 16 are positive for plasmid at 100 bp while Lanes 18, 33, 10, 4, 22 and 3 are negative for plasmid

TABLE 1: Antibiogram of plasmid containing *Salmonella* before and after treatment with sodium deodecyle sulphate

Salmonella identity*	Isolate	Inhibition zone diameter	
		Prior to treatment (mm)	After treatment(mm)
S10 (CN)		14	17
S19 (CN)		12	14
S22 (CN)		14	14
S24 (CN)		18	18
S25 (CN)		14	15
S34 (CN)		14	22
S36 (CN)		14	16
S39 (PEF)		18	23
S46 (LEV)		6	26
S47 (LEV)		6	28

* = isolates identity. CN= gentamicin, PEF= pefloxacin, LEV= levofloxacin
Plasmid cure occurred in *Salmonella* isolates 10, 34, 39, 46 and 47

However, after treatment with the curing agent, the zones of inhibition were 17 mm, 22 mm, 23 mm, 26 mm and 28 mm, respectively (TABLE 1).

DISCUSSION

In this study, the *Salmonella* isolates exhibited varying degrees of resistance to different antibiotics and this is in agreement with the report of Wegener *et al*. (1997). Thus, microbial resistance is the loss of sensitivity by a microorganism to an antimicrobial to which it was originally susceptible. The resistance can be acquired by mutations in chromosomal DNA or by the acquisition of extra-chromosomal genomic material by means of plasmids and transposons as reported by Vazquetz *et al.* (2005). The growing resistance of pathogenic bacteria to antimicrobial has raised the concern that the widespread use of antimicrobials in animal production may promote the development of resistance genes that can be transferred to bacteria that cause disease in humans as reported by Wegener *et al.* (1997).

This finding is consistent with Maloy *et al*. (1996), who reported plasmid cure using chemical agents such as acriflavin, acridine orange or sodium deodecyl sulfate solution. Also, the report of Posno *et al.* (1991) that antibiotics such as rifampin and mitomycin and DNA intercalating dyes such acridine orange and ethidium have shown cure of many plasmids is in agreement with the findings in this study. Due to plasmid loss, the bacterium no longer has the genetic advantage contained on the plasmid and this was reported by Fuji *et al.* (1992). The changes in bacteria characteristics and structure have been reported to be as a result of plasmid acquisition or plasmid elimination. The report of Poppe and Glyes, (1988) that the use of combinations like ethidium bromide, novobiocin and methylene blue resulted in curing six plasmids from *Salmonella* of avian origin is in agreement with the findings in this study. The variety of curing agents may have several different modes of action; they may affect the membrane potential, membrane permeability, protein lysis and the processing of DNA eliminating certain types of plasmids as reported by Boratynski and Viljunem, (1991).

In this study, plasmids were detected in eight of the 40 *Salmonella* isolates with sizes of 80 bp to 100 bp respectively. This is an indication that the resistance observed was plasmid mediated and this is in agreement with Kar (2008). Five *Salmonella* isolates that were initially resistant to

gentamicin, pefloxacin and levofloxacin before plasmid curing were found to be sensitive to these drugs after sodium deodecyl sulphate treatment. This indicates that resistance in these isolates was plasmid mediated, while those *Salmonella* isolates that were not still resistant after SDS treatment suggest that resistance was chromosomally mediated.

CONCLUSION

The antimicrobial resistance experienced in many poultry farms in the study area is likely due to the presence of plasmids as suggested by the detection of plasmids in this work.

ACKNOWLEDGEMENT

We sincerely appreciate Prof. Dennis Agbonlahor, of Lahore Research and Medical Diagnostic Centre Benin, who provided most of the materials and advice for the success of the work, Mr Ewere Sunday (Chief Technologist) of Veterinary Microbiology, Michael Okpara University of Agriculture, Umudike, and Mrs Ehiaghe Joy (Technologist) at Lahore for their technical support.

REFERENCES

AUSTIN, S.J. (1984). Bacterial plasmids that carry two functional centromere analysis are stable and are partitioned faithfully. *J. Bacteriol.* **158**: 742-745.

BAUMLER, A., NORRIS, T., LASCO, T., VOIGT, W., RESSBRODT, R., RABSCH, W., HEFFRON, F. and IRON, A. (1998). Novel outer membrane receptor characteristic of *Salmonella enteric*. *J. Bacteriol.* **180**: 1446-1447

BIRNBOIM, H.C and DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res.* **7**: 1513-1523.

BORATYNSKI, J and VILJUNEM, P. (1991). The susceptibility of conjugative resistance Transfer in gram Negative Bacteria to physiochemical and Biochemical agents. *Microbiol Rev.* **88**: 43 – 54.

CALLAGHAN, M. and SIMMONS, G. (2001). Outbreak of Salmonella typhimurium type 160 in Auckland linked to an umu function. *New Zealand Publ. Hlth. Rept.* **8**: 44-45.

DANIFOR BIOTECHNOLOGY, (2012). www.omicsonline,org/biotechnology 2012/

FUJI, N., YAMASHITA, Y., NAGASHIMA, M. and NAKANO, H. (1992). Induction of topiosomerase II medicated DNA cleavage by the plant naphthoquinones plumbagin and shikonin. *Antimicrob. Agents Chemoth.* **36**: 2589-2594.

GARNER, E.C., CAMPBELL, C.S., WEIBEL, D.B and MULLINS, R.D (2007) reconstitution of DNA segregation driven by assembly of a prokaryotic homologue. *Sci.* **315**: 1270-1274.

GERDES, K., LARSEN, J.E. and MOLIN, S (1985). Stable inheritance of plasmid requires two different loci. *J. Bacteriol.* **101**: 292-298.

GILBERT, S., LAKE, R., CRESSEY, P., HUDSON, A. and KING, N. (2010). Risk profile: *Salmonella* (Non typhoidal in pork and pork products). Institute of Environmental Science and Research Limited. www.foodsafety.gov.nz/elibrary

GROHMANN, E. (2003) national center for biotechnology information.www.ncbi.nlm.gov.

GUINEY, D.G., FANG, F.C., KRAUSE, M. and LIBBY, S. (1994). Plasmid-mediated virulence genes in non-typhoid *salmonella serovars. Microb. Lett*. **124**: 1-9.

GULIG, P.A., DANBARA, H., GUINEY, D.G., LAX, A.J., NOREL, F. and RHEN, M. (1993). Molecular analysis of Spv virulence genes of *Salmonella* virulence. *Mol. Microbiol*. **7:** 825-830.

HAYES, A (2000). The partition system of multidrug resistance plasmid included a novel protein that epitomizes an evolutionary district subgroup of the par 'A' super family. *Mol. Microbiol.* **37**: 528-541.

KAR, A. (2008). Characterization, classification and taxonomy of microbes in pharmaceutical microbiology, new age international Publishers Ltd. Ansari Road, Daryaganj, New Delhi, PP: 62-111

MALOY, S. R., STEWART, V.J. and TAYLOR.R.K. (1996). Genetic analysis of pathogenic bacteria. Cold Spring Harbor Laboratory press, Cold Spring Harbor, N. Y.

MANJISTHA, S. and STUART, A. (2011) prevalence and significance of plasmid maintenance functions in the virulence plasmids of pathogenic bacteria. *Infect. Imm.* **7**: 2502-2509.

NWIYI, P., CHAH, K.F. and SHOYINKA, S.V.O. (2016). Molecular detection of *Salmonella* isolated from poultry farms in Abia and Imo states, southeast, Nigeria. *Inter. J. Current Microb. Appld. Sci.* **5** (7): 961-968

OLIVEIRA, S.D., ROCLENBUISCH, I.R., ROCHA, S.L. and CANAL, C.W. (2003). Evaluation of selective and non-selective enrichment PCR procedures for *Salmonella* detection. *Lett. Appld. Microbiol.* **36**: 25-35.

OU, J. T., BARON, L. S., DAI. X. Y. and LIFE, C. A. (1990). The virulence plasmids of salmonella serovars typhmurium, choleraesuis, Dublin, and enteritidis, and the cryptic plasmids of Salmonella serovars Copenhagen and sendai belong the belong to the same incompatibility group, but not those of Salmonella serovars Durban Gallinarum, Infantis and Pullorum. *Microb. Pathog.* **8:** 101-107.

OU, J.T (1993). The 90kilobase pair virulence plasmid of Salmonella serovar Typhimurum coexisys in strains with a plasmid of the 23 incompartibility groups. *Microb. Pathog.* **15**: 237-242.

POPPE, C. and GYLES, C.L. (1988). Tagging and elimination of plasmids in *Salmonella* of avian origin. *Vet. Microb.* **18**:73-87

POSNO, M., LEER, R. J., VAN LUIKJ, N., VAN GIEZEN, M. J., HWUVELMANS, P. T., LOKMAN, B. C. and POUWELS, P. H. (1991). Incompatibility of lactobacillus vectors with replicons derived from small cryptic lactobacillus plasmids and segregation introduced vectors. *Appld. Env. Microbiol.* **57**: 1822 – 1828.

SUMMERS, D.K., BETON, C. and WITHERS, H.L (1993). Multicopy plasmid instability. The dimer catastrophe hypothesis. *Mol. Microb.* **8**: 1031-1038.

VAZQUEZ, N. J., CORDOBA, B. C., LOPEZ, N. Y. and MANCERA, M. A. (2005). Identification del gene da la integrasa tipo 1 y perfil da Resistencia antimicrobiana em *Salmonella Enteritidis.* Cuajimalpa (cited 2005 Nov 24). Available from: www,vet-uy.com/articulos/artic_micro/001/micro001.htm.

WEGENER, H. C., BAGER, F. and AARESTRUP, F. M. (1997). Vigilancia da Resistencia aos antimicrobianos no homem, nos produtos alimentares e no gado na Dinamarca. *Euro. Surveill.* **3**(2):17-19

WONG, T.L., NICOL, C., COOK, R. and MACDIARMID, S. (2007). *Salmonella* in uncooked retail meats in New Zealand. *J. Food Protect.* **70** (6): 1360-1365.