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GENETIC RELATEDNESS OF ESBL AND NON-ESBL *SALMONELLA* TYPHIMURUM ISOLATED FROM POULTRY BIRDS AND POULTRY HANDLERS IN NASARAWA STATE, NIGERIA.

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ABSTRACT

Multidrug resistant salmonellosis infection is an important global public health problem particularly in sub-Saharan Africa, where it commonly manifests as gastroenteritis and/or bloodstream infections in both children and adults. *Salmonella* Typhimurium has a broad-host-range and poultry birds are an important reservoir. The unrestricted use of Beta-lactams has generated resistance through a gene encoded Extended Spectrum Beta-lactamase (ESBL) enzyme production, which impedes the successful therapy of infections. This study aims at investigating the genetic relatedness of ESBL and non-ESBL S. Typhimurium strains isolated from humans and Poultry birds. A total of 19 *S.* Typhimurium isolated from Poultry (droppings, flesh, feeds) and handlers (feces, hand swabs) in a previous study were used. These had earlier been identified using pre-enrichment and selective enrichment culture media. Further identification was carried out using conventional biochemical screening tests and slide serology tests using polyvalent antisera. Antibiotic susceptibility testing including the Double Disk Synergy test (DDST) for screening of ESBL production was carried out as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines. This study also employed the PCR-RFLP method which involves the simple restriction digestion of purified 16S rRNA of *Salmonella* and variation in the banding patterns revealed their genetic relatedness. All the strains were found to share identical molecular base profiles hence are genetically related variants. The selective pressure due to antibiotic mismanagement in Poultry farming is the likely trigger of ESBL production observed to occur only among the *S.* Typhimurium strains from poultry origin. This calls for a holistic approach in antibiotic stewardship.

Keywords: Genetic relatedness, PCR-RFLP, 16S rRNA gene, ESBL, *salmonella* typhimurium, Nasarawa, poultry.

INTRODUCTION

Salmonella enterica is a zoonotic pathogen which can readily pass from animal to man through the consumption of contaminated foods (Cosby *et al*., 2015; Card *et al*., 2016). Majority of over 2650 recognized serotypes of *Salmonella* infect both humans and animals worldwide with signs ranging from fever, abdominal cramps, vomiting, diarrhea and death (Guibourdenche *et al*., 2010; Scallan *et al*., 2011; Issenhuth-Jeanjean *et al*., 2014). Poultry is an important source of antimicrobial resistant bacteria including the ESBL-producing *Salmonella* (Oyinloye *et al*., 2011). Production of ESBLs is a significant resistance mechanism that impedes the antimicrobial therapy of infections caused by some *Enterobacteriaceae* and is a serious threat to the currently available antimicrobial options (Shaikh *et al*., 2015). *Salmonella* strains, affecting both humans and animals exhibiting resistance to many of the currently available antibiotics used in therapy have emerged and are increasing in frequency with more hospitalization. This has serious implications for farmers, consumers of food animals and the public health (Lu *et al*., 2014; Mukherjee *et al*., 2019). Therefore, identifying and typing these strains are key steps in the successful therapy of salmonellosis infections as well as for epidemiological purpose (Turki *et al*., 2014).

Multiple typing methods are available and used for discriminating microorganisms at strain level based on either phenotypic or genotypic traits. The phenotypic method includes biotyping, serotyping, phage typing, antibiotic susceptibility testing, mass spectrometry (MS) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of cellular-extracellular components. Whereas the genotypic methods target the nucleic acid, and involves use of the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), Pulsed Field Gel Electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), ribotyping, multilocus sequence typing and multiplex PCR (Neslihan *et al*., 2018). The current gold standard for molecular typing is Pulse-Field Gel Electrophoresis (PFGE), which can provide discrimination between similar serotypes and is the basis for PulseNet surveillance (Soler-García *et al*., 2014). Unfortunately, PFGE is a laborious, time-consuming, and expensive method. A sub-typing method should be rapid, robust, portable and sensitive. It should be able to reliably differentiate epidemiologically unrelated strains from each other and group all isolates associated with the same source without disrupting their classification into subspecies and serovars (Soler-García *et al*., 2014). Such a sub-typing system would also need to be within budgets of laboratories, researchers and students especially in the developing Countries. It is also required to be less time consuming for prompt identification and commencement of therapy in the event of an outbreak.

Polymerase Chain Reaction-Restriction fragment length polymorphism (PCR-RFLP) is a variation of RFLP which is one of the easiest ways to study diversity of microbes (Chatterjee, 2019). The technique uses the simple restriction enzyme digestion of purified DNA from bacteria, and variation in the banding pattern in the digestion reveals the genetic diversity. The 16S rRNA and 23S rRNA are the most widely used molecular chronometers. For adequate discrimination, the amplified region or gene needs to have a variable region flanked by conserved regions to allow PCR amplification and generation of different restriction patterns after cutting with restriction enzymes (Soler-García *et al*., 2014). PCR-RFLP has been successfully used for the study of diversity of *V. cholerae* strains (Chowdhury *et al*., 2010). In a recent study, it was stated that RFLP can be used to study taxonomy of *Y. pestis*, *S. aureus* coagulase gene diversity from food products, as well as diversity in the plasmid from *E. coli* which was isolated from water source (Qi *et al.* 2016 ; Dallal *et al.*2016) as cited in Chatterjee & Rajal (2019). Neslihan *et al*. (2018) also used PCR-RFLP to determine the genetic variability of 38 foodborne *Salmonella* isolates that were previously identified by biochemical tests.

Backyard poultry farms are a common sight in our study area and are mostly practiced under low levels of hygiene. There is a risk of infection and cross contamination with antibiotic resistant *S*. Typhimurium strains harbored in the guts of these birds with food, beverages, water, fruits and vegetables consumed by Man. Successful antibiotic therapy of such infections can be achieved when the variant of the strains implicated are properly identified and typed by adopting one of the phenotypic and/or genotypic typing methods. To the best of our knowledge, no study has been done in north central Nigeria using the PCR-RFLP method to determine the genetic relatedness between the ESBL and non-ESBL *S.* Typhimurium strains from poultry and their handlers as an attempt to adopt a cheap and reliable means of prompt laboratory diagnosis of multidrug resistant or ESBL salmonellosis infections.

The study aimed at using PCR-RFLP method to study the genetic relatedness of *Salmonella* Typhimurium strains isolated from poultry birds and their handlers which were previously exposed to critically important antibiotic groups used for therapy in both human and veterinary medicine.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

A total of 19 multidrug resistant (MDR) *S*. Typhimurium isolates earlier characterized from a previous study (Ibrahim *et al*., 2019) were selected for this study. However, out of the selected isolates, 16 were ESBL- producers from poultry birds, while the remaining 3 were non-ESBL producers from poultry farm workers. The isolates were stored in sterile nutrient agar slants at 4ºC. The *Salmonella* strains were grown overnight (18 h) under aerobic conditions at 37 °C in XLD (Oxoid Ltd, Hampshire, UK).

Primers

The 16S rRNA primer used in this study as listed in Table 1.

Table 1: Primer for 16S rRNA gene of *Salmonella*

Primer	Sequence (5' – 3')	Amplicon length (bp)	Reference
16S rRNA	F: AGAGTTTGATCMTGGCTCAG	27	Jiang *et al*., 2006
	R:CGGTTACCTTGTTACGACTT	1492	

Key: bp = base pairs

DNA extraction (Boiling method): Bacterial culture was inoculated into sterile Luria-Bertani (LB) broth and incubated at 37ºC for 8 h. Five millilitres of the LB broth culture containing the bacterial isolates was spun at 14000 rpm for 3 min. The cells were resuspended in 500 µl of normal saline and heated at 95ºC for 20 min in the heating chamber. The heated bacterial suspension was cooled on ice and thereafter spun for 3 min at 14000 rpm. The supernatant containing the DNA was transferred into a 1.5-ml micro centrifuge tubes and stored at -20ºC for subsequent experimentations (Ghorbani-Dalini *et al*., 2015).

DNA Quantification: The extracted genomic DNA was quantified using the NanoDrop 1000 spectrophotometer by placing a drop (approximately 2 µl) on the sample space and analysed using the NanoDrop 1000 software. For DNA concentration, absorbance readings were performed at 260 nm (A_{260}) and the readings were observed to be within the instrument's linear range (0.1 – 1.0). DNA purity was estimated by calculating the A_{260}/A_{280} ratio and this was done by the spectrophotometer's computer software (where A_{260}/A_{280} ratio ranges from 1.7 – 1.9).

Restriction Fragment Length Polymorphism (RFLP) analyses

16S rRNA gene Amplification: The 16s rRNA region of the rRNA genes of the bacterial isolates were amplified using the universal 16s rRNA gene primers listed previously (Table 1) in a thermal cycler at a final volume of 25 µl for 35 cycles. The PCR mix included: X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, $MgCl_2$), the primers at a concentration of 0.2M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 min; denaturation, 95ºC for 30 sec; annealing, 52ºC for 30 sec; extension, 72ºC for 30 sec for 35 cycles and final extension, 72ºC for 5 min. The product was resolved on a 1.5% agarose gel at 120V for 20 min and visualized on a UV transilluminator.

Restriction enzyme digestion of the 16S rRNA gene: The method of Lacher *et al*. (2006) was adopted for RFLP to identify genetic diversity of the *S.* Typhimurium isolates. To achieve species–specific discriminatory patterns, a 5 µl aliquot of 16S rRNA gene PCR amplicons were enzymatically digested with 10U/µl of *Eco 471* and *BsGr* separately (AvaII – ThermoFisher Scientific) in a final volume of 25 µl at 37ºC for 6 h. The restriction fragments were separated on 2% agarose gel electrophoresis in TBE buffer for about 30 min at 120V and visualized by staining with 0.5 µg/ml of ethidium bromide.

Determination of genetic relationship between ESBL and Non-ESBL *S.* Typhimurium strains from Poultry and Poultry farm workers

The genetic relationship between *S.* Typhimurium isolates from Poultry and Poultry farm workers was further determined by an analysis of the PCR-RFLP profiles presented as a Dendrogram.

RESULTS

Analysis of the genetic relatedness between the ESBL and Non-ESBL *S.*Typhimurium strains using PCR-RFLP endonulease enzymes generated molecular profiles which are summarized as shown in Tables 1-2 and Plates 1-2 respectively.

Table 1: PCR-RFLP Profiles of *Eco* 471 endonuclease digestion of 16S rRNA of the S.Typhimurium strains isolated from Poultry (droppings, feeds, flesh) and Poultry farm handlers (feces and hand swab) in Nasarawa State

Isolate ID	No. of Isolates	*Eco* 471 Profiles	No. of bands	RFLP Pattern/Variant
P1-P5, P7-P9, P15, P16	10	380-700	2	A
H1, H2, H3	3	380-700	2	A
P14	1	380-490-750	3	B
P6,P10	2	350-750	2	A
P11, P12, P13	3	380-500	2	A
Total	19			

Key: ID = Identity, P = Poultry, H= Handlers

Table 2: PCR-RFLP Profiles of *BsGr* endonuclease digestions of 16S rRNA of the S.Typhimurium strains isolated from Poultry (droppings, feeds, flesh) and Poultry farm handlers (feces and hand swab) in Nasarawa State

Isolate ID	No. of Isolates	*BsGr* Profiles	No. of bands	RFLP Pattern/Variant
P1-P10,P15,P16	12	200-300-500	3	B
H1,H2,H3	3	200-300-500	3	B
P14	1	300-500	2	A
P11,P12,P13	3	200-400-500	3	B
Total	19			

Key: ID = Identity, P = Poultry, H = Handlers

Gel Pictures for the PCR-RFLP

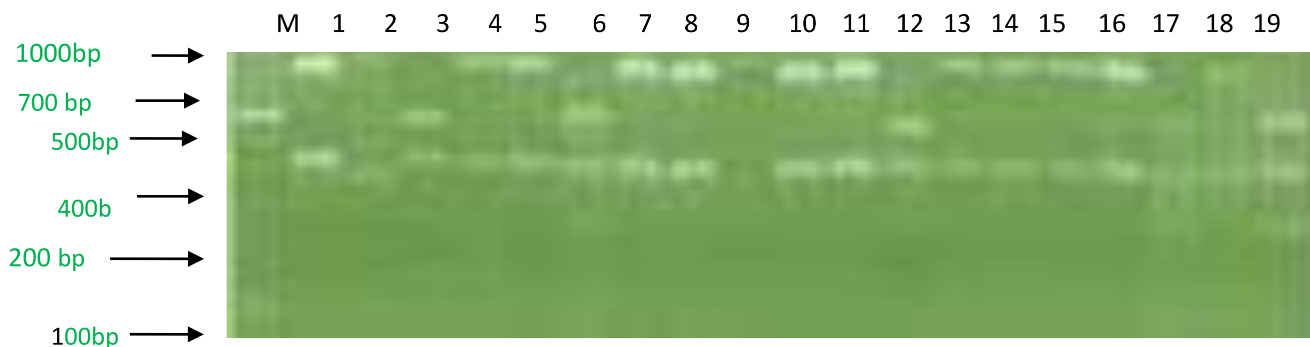

Plate 1: PCR-RFLP profiles of 16S rRNA gene of *S.*Typhimurium showing different bands pattern after digestion with *Eco*471. Lane 1, 4, 5, 7, 8, 10-11, 13-16, 18-19 (380-700 bp) represents isolates H1-H3, P1-P5, P7- P9, P15-16 Lane 2 and 9 (350-750bp) represent isolates P6 & P10, Lane 3, 12, 17 (380-500bp) represent isolates 11,12 &13, Lane 6 (380-490 -750)represents isolate P14, while M represents a 100bp ladder.

Key: P = Poultry, H = Handlers

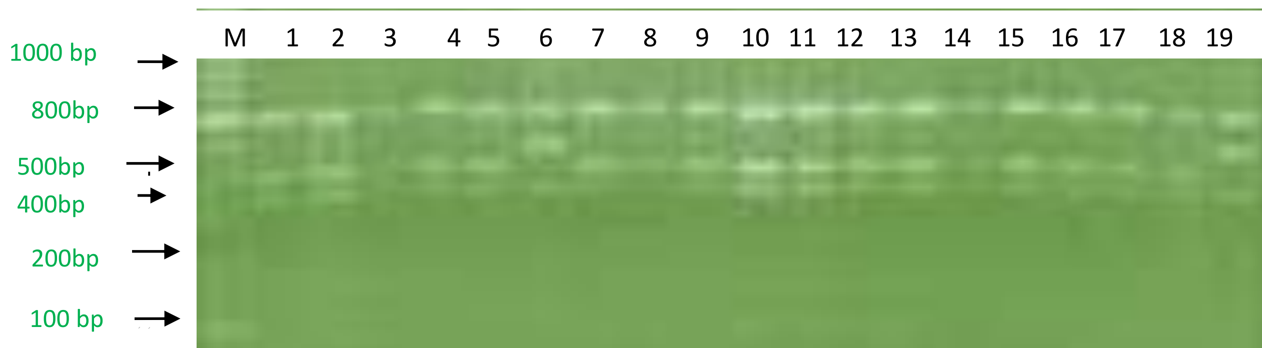

Plate 2: PCR-RFLP profiles of 16S rRNA gene of *S.*Typhimurium after digestion with *Bs*Gr restriction endonuclease. Lane 1- 5, 7-17 and 18 (200-300-500 bp) represents isolates H1-3, P1-P10, P12, P15, P16, Lane 8 (300-500 bp) represents isolate P14, Lane 6 and 19 (200-400- 500 bp) represents isolates P11& P13 while M represents a 100 bp molecular ladder.

Key: P = Poultry, H =Handlers

DISCUSSION

Salmonella enterica serotype Typhimurium is documented to be a prototypical broad-host-range serotype as a result of it s ability to colonize and cause infections in a vast majority of animal species; humans, livestock, domestic fowl, rodents and birds (Rabsch *et al*., 2002; Feasey *et al*., 2012; WHO, 2018). Serotype *S*. Typhimurium variants with a narrow host range have also been documented (Rabsch *et al*., 2002). The PCR-RFLP method employed for this study was able to discriminate the variants of ESBL and non-ESBL *S.*Typhimurium strains isolated from both poultry birds and their handlers. All the non-ESBLs from poultry handlers and the ESBLs from poultry with the exception of two strains had similar number of molecular bands and also shared identical lengths of cleavage fragments. In addition, they occupied the same position in the cluster following analysis of the dendrogram. All these signify that they are genetically related variants with a common source of contamination. This is similar to the findings of Chenggang *et al*. (2017) in China where majority of *Salmonella* isolates from different sources possessed identical molecular finger prints, implying relatedness and a zoonotic transmission.

Similarly, results of the *BsGr* endonuclease digestions revealed that all the ESBL and non-ESBL *S.*Typhimurium strains shared specific RFLP pattern of finger prints; 200bp, 300bp and 500bp indicating a characteristic sequence of DNA. The results of their pattern similarities were further presented as a dendrogram which also grouped the fingerprints into same cluster hence, implies that the isolates are genetically related variants having a common source of contamination with a zoonotic characteristic. Our study finding indicated that the *BsGr* endonuclease gave a better discrimination and produced a better digestion result with multiple interpretable PCR-RFLP patterns because this enzyme had more restriction sites for the 16S r RNA gene compared to the *Eco*471 enzyme.

Interestingly, the ESBL *S*. Typhimurium variants were restricted to Poultry birds. It is likely that the selective pressure due to persistent exposure to antibiotics arising from its misuse and overuse triggered the evolvement of these variants. Antibiotics, particularly the beta-lactam groups are known to be incorporated in feed additives to serve as growth enhancers and or prophylaxis against infections (Landers *et al*., 2012) in food animals. Colonization and infection of humans and othern animals with these particular variants can hamper the successful therapy of severe non-typhoidal salmonellosis infections using extended spectrum cephalosporins licensed for use in both human and veterinary medicine. In addition, their antibiotic resistance determinants can easily be transferred to other bacteria which can also stall the therapy of infections they cause. The occurrence of these variants of ESBL and non-ESBL S. Typhimurium in our study area further highlights the role of proper organism identification, antibiotic susceptibility testing and typing of bacterial strains as key steps in successful therapy of salmonellosis infections as well as for epidemiological purpose.

The findings in the present study using PCR-RFLP with double endonuclease digestions agrees with the studies of Khaki *et al*. (2013), Dilmaghani *et al*. (2010) and Zaki *et al*. (2009). The studies of Sumithra *et al*. (2014), Khaki *et al*. (2013) and Matsuie *et al*. (2001) however, suggest that PCR-RFLP using more than one endonuclease and genes gives good typeability and increases the differentiating power. Although the studies of Neslihan *et al*. (2018) concluded that PCR-RFLP is a good typing method but lacked sufficient power of discrimination which is at variance with the present study findings. Nevertheless, researchers have employed PCR-RFLP for serotyping and analysis of diversity in microorganisms using different genes and double combinations of endonucleases to which they obtained varying results (Moradi *et al*., 2015).
The report of Akbarmehr *et al*. (2010) is contrary to our study findings probably due to differences in the endonuclease enzymes employed for both studies.

Further studies employing different sets of enzymes and genes in order to create more choices for a successful PCR-RFLP to study diversity among *Salmonella* serotypes is recommended.

CONCLUSION

All the *S*. Typhimurium strains were found to share identical molecular base profiles hence are genetically related variants. However, the selective pressure due to beta-lactam antibiotic misuse and overuse in Poultry farming triggered ESBL production which was observed to occur among the *S*. Typhimurium strains isolated from poultry source (droppings, flesh and feeds). This calls for a holistic approach in antibiotic stewardship.

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