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-Garcia 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-Garcia 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, 1999). 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 4°C. 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 R: CGTTACCCTTTGTTACG | 27 | Jiang et al., 2006 |
|         |                   | 1492                |           |

Key: bp = base pairs

**DNA extraction (Boiling method):** Bacterial culture was inoculated into sterile Luria-Bertani (LB) broth and incubated at 37°C 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 95°C for 20 min in the heating chamber. The heated bacterial suspension was cooled on ice and thereafter spun at 3 min at 14000 rpm. The supernatant containing the DNA was transferred into a 1.5-ml micro centrifuge tubes and stored at -20°C 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 (A260) and the readings were observed to be within the instrument’s linear range (0.1 – 1.0). DNA purity was estimated by calculating the A260/A280 ratio and this was done by the spectrophotometer’s computer software (where A260/A280 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, MgCl2), the primers at a concentration of 0.2M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 min; denaturation, 95°C for 30 sec; annealing, 52°C for 30 sec; extension, 72°C for 30 sec for 35 cycles and final extension, 72°C 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 471I and BsGr separately (AvaiI – ThermoFisher Scientific) in a final volume of 25 µl at 37°C 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 endonuclease 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 Eco471. 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
DISCUSSION

*Salmonella enterica* serotype Typhimurium is documented to be a prototypical broad-host-range serotype as a result of its 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 *Bs*Gr 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 *Bs*Gr 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 Eco471 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 other 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), Dilmagnani et al. (2010) and Zaki et al. (2009). The studies of Sumithra et al. (2014), Khaki et al. (2013) and Matsue 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 Akbarneh 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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