Detection of some resistance genes in *Salmonella* isolated from Poultry farms in Abia and Imo States, Southeastern Nigeria

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**SUMMARY**

Sixteen *Salmonella* Gallinarum and 24 *Salmonella* Pullorum strains isolated from chickens were screened for resistance to 11 antibacterial agents using the disc diffusion method. Five of the *Salmonella* Gallinarum and five of *S.* Pullorum strains resistant to streptomycin, gentamicin, tetracycline and sulfamethoxazole/trimethoprim were screened for presence of strA/strB, aac (3)-II, aac (3)-IV, tetA and tetB and sul 1 (dfr/A) and sul 3 (dfr/G) resistance genes. A singleplex PCR with resistance gene specific primers was used to ascertain the presence of the target resistance gene. All the *Salmonella* isolates studied were resistant to ampicillin while 95% were resistant to tetracycline and streptomycin. None of the isolates were resistant to ofloxacin and ciprofloxacin. Three of the sulphamethaxazole/trimethoprim-resistant isolates haboured dfrA and dfrG genes while one of the gentamicin-resistant isolates was positive for aac (3)-IV genes. None out of the 10 streptomycin and tetracycline resistant isolates harbored any strA/strB, tetA and tetB genes. In addition, none of the 10 gentamicin resistant isolates harbored aac (3)-IV genes. The high resistance rates recorded in this study may be attributed to indiscriminate use of antibacterial agents.

**Key words:** *Salmonella*, antimicrobials, resistance genes, Polymerase chain reaction, Southeastern Nigeria

**INTRODUCTION**

The Genus *Salmonella* comprises of Gram-negative, non-sporing rods (2-4 x 0.5um) that do not have capsules and are in the Family *Enterobacteriaceae* (OIE Manual, 2006), some of which are pathogenic. Members of this Genus, except *S.* Pullorum and *S.* Gallinarum, are motile and have long flagellae (OIE Manual, 2006). They grow readily on ordinary media and most agars, forming large, thick, grayish white, domed shaped colonies on deoxycholate citrate media. All ferment glucose but not lactose, all reduce nitrates to nitrates and all can survive for several months away from the host (Hossain *et al.*, 2006). Salmonellae are found in the intestinal tract of humans and many animals, including domestic animals, such as chickens and cattle. Chickens are natural host for both *S.* Pullorum and *S.* Gallinarium (Snoeyenbos, 1991). *Salmonella* is a well-known genus because members of the genus have the
ability to cause disease. More than 2,500 serotypes of *Salmonella* have been identified, only about 10% of these have been isolated from poultry (Gast, 1997). Incidences of occurrence of *Salmonella* have been traced to only few forms, mostly *S. Pullorum*, *S. Typhimurium* and *S. Enteritidis* (Breslow 2002). Salmonellosis is the name of a group of infectious diseases caused by *Salmonella* serotypes and include fowl typhoid, typhoid fever, paratyphoid fever, and food poisoning (OIE Manual, 2006). These infections can be reduced through proper hygiene and personal and social responsibility.

Microbial resistance is the loss of sensitivity by a microorganism to an antimicrobial to which it was originally susceptible. This resistance can be acquired by mutations in chromosomal DNA or by the acquisition of extra- chromosomal genomic material by means of plasmids and transposons (Vazquez et al., 2005). The growing resistance of pathogenic bacteria to antimicrobials has raised the concern that the widespread use of antimicrobials in animals’ production may promote the development of resistant bacteria or resistance genes that can be transferred to bacteria that cause disease in humans (Wegener et al., 1997). A Major public health problem has been the emergence and spread of antimicrobial resistance in bacteria populations. There is a significant increase in the frequency of isolation of bacteria that were once sensitive to routine drugs, but are now resistant to nearly all drugs in the market (Nogueira et al., 1999).

The emergence and persistence of antibiotic resistance in *Salmonella* spp. continue to pose serious risks to human and animal health (Joseph et al., 2008).

Resistance to antimicrobial drugs was first reported in the studies published in 1907 by Paul Ehrlich, who recorded the emergence of trypanosomes resistant to rosaniline chemotherapy.

The emergence of bacterial resistance was also recorded after sulfonamide and penicillin started to be used in veterinary and human medicine in the 1940s (Quinn et al., 2001) There is a growing concern as to the proper use of antibacterials. One negative aspect of the use of antimicrobial is the selection of multi- resistant microorganism, limiting the therapeutic possibilities, and increasing not only the lethality rates, but also treatment costs (Nogueira et al., 2005). The presence of plasmids of high molecular weight (50 to 100kb) has been demonstrated, where genes encoding for toxins are found, as well as genes that confer multi-resistance to antimicrobials (Vazquez et al., 2002).

Previous study has shown that antimicrobial use in animal production systems may lead to the increase of drug- resistance among animal pathogen (Heider et al., 2009; Mann et al., 2011; Morley et al., 2011).

This study was conducted to determine the antibacterial resistance profile and resistance genes in *Salmonella* Gallinarum and *S. Pullorum* from chickens in Abia and Imo States, Nigeria.

**MATERIALS AND METHODS**

**Bacterial isolates**

Stocked cultures of *Salmonella* Gallinarum (16 strains) and *Salmonella* Pullorum (24 strains) were used for the study. The isolates were obtained from chickens in Abia and Imo States (Nwiyi et al., 2016).

**Antimicrobial resistance profile**

Antimicrobial resistance profile of the Salmonella isolates was determined by the disc diffusion method of Kirby Bauer (1966) as described by the Clinical Laboratory Standard Institute (CLSI, 2014). Each *Salmonella* isolate was enriched in nutrient broth for 15 minutes at 37°C before swabbing on to the surface of dried plates of Mueller-Hinton agar (MHA). A total of 11 antimicrobial agents were used for the study. The antibiotics used was obtained from Oxoid, (United Kingdom) and their concentration were as follows: Ampicillin (30µg), tetracycline (25µg), gentamicin (10µg), perfloxacin (10µg), ofloxacin.
(30µg), sulfamethoxazole/trimethoprim (30µg), streptomycin (10µg), ciprofloxacin (5µg), levofloxacin (30µg), nalidixic acid (30µg) and ceftriaxone (10µg). The antibiotic disks were placed on the agar surface, sufficiently separated from each other so as to avoid overlapping of inhibition zones. Each plate carried a maximum of six discs and each test was performed in duplicate. After 30 seconds of pre-diffusion, the plates were incubated at 37°C for 24 hours after which the diameter of inhibition zones were measured with a metre rule and the inhibition diameter for each isolate and each antimicrobial agent was calculated to the nearest whole number. For each antimicrobial agent, the isolates were recorded as sensitive or resistant according to the interpretation guidelines of CLSI (2014).

Detection of antimicrobial resistance genes in the *Salmonella* isolates

Ten strains (five *Salmonella* Gallinarum and five *Salmonella* Pullorum) resistant to gentamicin, sulfamethoxazole-trimethoprim, streptomycin and tetracycline were selected and screened for presence of genes conferring resistance to streptomycin (*str*/A/B), gentamicin [aac (3)-ii and aac(3)-iv], tetracycline (*tet*A and *tet*B) and sulfamethoxazole/trimethoprim (*sul* 1 (*dfr*A) and *sul* 3 (*dfr*/G)). Each test isolate was grown on MacConkey agar and genomic DNA was extracted from the colonies using the boiling method and in accordance with the protocol of Danifor Biotechnology (2012). The target resistance genes were detected using singleplex polymerase chain reaction (PCR). The cycling conditions and primer sequence were as described by Ma et al. (2007). The PCR was performed in 30µl volumes containing 3µl of buffer (100mmol/L Tris-HCl (pH 9), 1.5mmol/L MgCl₂, 500mmol/L KCl, 0.1% gelatin), 100mmol/L concentration each of dATP, dGTP, dGTP and dCTP, 10pmol of each primer, and 0.9U of Taq DNA polymerase (Inqaba Biotechnical Industries Ltd, South-Africa), with 2.0µL of template DNA. The primer sequence (Table 1) and cycling conditions used in this study are: Initial denaturation step at 95°C for 5min, followed by annealing at 35°C for 30 sec and a final elongation at 72°C for 2min. The amplified genes 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 gel was visualized and photographed under transilluminator ultraviolet (UV) light with gel documentation (MB) Fermenters USA.

### TABLE I: Primer set for resistance genes detection

<table>
<thead>
<tr>
<th>Resistance genes</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>str*/A/B-F</td>
<td>ATGGTGGACCCCTAAACTCT</td>
</tr>
<tr>
<td>str*/A/B-R</td>
<td>CATCTAGGATCGAGACAAAG</td>
</tr>
<tr>
<td>aac(3)-11-F</td>
<td>ACGCAGGAAAGCAATACCGA</td>
</tr>
<tr>
<td>aac(3)-11-R</td>
<td>TAACCTGAAAGGCCTCGAAGA</td>
</tr>
<tr>
<td>aac(3)-IV-F</td>
<td>TGCTGGTCCACAGCTTTC</td>
</tr>
<tr>
<td>aac(3)-IV-R</td>
<td>CGGATGCGAGGAGATCAA</td>
</tr>
<tr>
<td>tet*A-F</td>
<td>GCTACATCCTGCTGGTCTTC</td>
</tr>
<tr>
<td>tet*A-R</td>
<td>CATAGATCGCCGTAAGAGG</td>
</tr>
<tr>
<td>tet*B-F</td>
<td>TTGGTTAGGCGGCGAGTGG</td>
</tr>
<tr>
<td>tet*B-R</td>
<td>GTAATGGGCAATAACACCG</td>
</tr>
<tr>
<td>sul1(<em>dfr</em>/A)-F</td>
<td>CGGCGTGGCTACGTGAACG</td>
</tr>
<tr>
<td>sul1(<em>dfr</em>/A)-R</td>
<td>GCCGATCGCGTGAAGTTCG</td>
</tr>
<tr>
<td>sul3(<em>dfr</em>/G)-F</td>
<td>CAACCGAAGTTGGGCGTTGG</td>
</tr>
</tbody>
</table>

**RESULT AND DISCUSSION**

The resistance profile of *Salmonella* isolates to 11 antimicrobial agents is presented in Table II. *Salmonella* Pullorum isolates were resistant to ampicillin, streptomycin and tetracycline while 91.7, 58.3 and 50.0% were resistant to nalidixic acid, sulfamethaxazole/trimethoprim and perfloxacin, respectively while *Salmonella* Gallinarum isolates were all resistant to ampicillin and gentamicin; while resistance to streptomycin, tetracycline and nalidixic acid was demonstrated by 87.5, 87.5...
sul3(dfr/G)-R GCTGCACCAATTGCCTGAACG

F=Forward, R=Reverse

In this study, all the Salmonella isolates were evaluated for resistance against five classes of antimicrobial agents namely: the aminoglycosides, quinolones, cephalosporins, sulphonamides and tetracycline. Resistance to antibiotics seen among Salmonella strains has become a global problem (Baggesen et al., 2000). Lee et al. (2003) in his study reported Salmonella resistance to many antibacterial agents in many countries including Nigeria. The Salmonella strains in this study, demonstrated high resistance rates to the

**TABLE 2:** Antibacterial resistance profile of Salmonella Pullorum (n=24) and Salmonella Gallinarum (n=16) isolated from chickens in Abia and Imo states

<table>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>S. Pullorum (n=24)</th>
<th>S. Gallinarum (n=16)</th>
<th>Total (n=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No resistant (%)</td>
<td>No resistant (%)</td>
<td>No resistant (%)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>24 (100)</td>
<td>16 (100)</td>
<td>40 (100)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>24 (100)</td>
<td>14 (87.5)</td>
<td>38 (95)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>24 (100)</td>
<td>14 (87.5)</td>
<td>38 (95)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>22 (91.7)</td>
<td>12 (75)</td>
<td>34 (85)</td>
</tr>
<tr>
<td>Sulfamethoxazole/trimethoprim</td>
<td>14 (58.3)</td>
<td>12 (75)</td>
<td>26 (65)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>12 (50)</td>
<td>16 (100)</td>
<td>28 (70)</td>
</tr>
<tr>
<td>Perfloxacin</td>
<td>12 (50)</td>
<td>4 (25)</td>
<td>16 (40)</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0 (0)</td>
<td>4 (25)</td>
<td>4 (10)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0 (0)</td>
<td>2 (12.5)</td>
<td>2 (5)</td>
</tr>
</tbody>
</table>

**Figure 1:** Polymerase chain reaction results for Salmonella isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Sample 46 is positive for aac (3)-II resistance gene with band at 100bp while samples 34 and 36 are negative for aac (3)-II resistance gene. NC is a no DNA template control

**Figure 2:** Polymerase chain reaction results for Salmonella isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 18, 22 and 47 are positive for Sul-1-(dfr/A) resistance gene with bands at 150bp. NC is a no DNA template control
commonly available antimicrobial agents (ampicillin, tetracycline, streptomycin, and nalidixic acid). The resistance to commonly available antimicrobials may be a reflection of their indiscriminate use in poultry used in veterinary practice (Smith et al., 2011). This wide and indiscriminate use may also explain the emergence of resistance to this class of antimicrobial agents. Ofloxacin and ciprofloxacin are not used in veterinary practice in the study area. This may explain the reason why the Salmonella strains were not resistant to these antimicrobial agents. Sulfamethaxazole/trimethoprim and gentamicin have shown evidence of resistance due to poor response of Salmonella to these drugs on usage by farmers Ojo et al. (2012).

Ampicillin resistance was observed in all the isolates and this is in agreement with the findings of Deekshit et al. (2012) who reported 100% resistance to ampicillin. The frequent use of ampicillin for treatment by most poultry farmers as well as the low dose applied may be responsible for the high resistance recorded in this study and this agrees with the observation by Suresh et al. (2006).

Resistance of Salmonella to streptomycin in this current study was 100% and this is in agreement with Sultana et al. (1992), but in disagreement with Cardoso et al. (2006) and Carraminana et al. (2004). Cardoso et al. (2006) in his study on Salmonella in Pakistan reported that Salmonella were resistant to streptomycin at 64.2%, while fluoroquinolones (levofloxacin and ciprofloxacin) are now widely prescribed and

The fluoroquinolones (levofloxacin and ciprofloxacin) are now widely prescribed and used in veterinary practice (Smith et al., 2011). The high resistance rate to streptomycin observed in the present study may be due to indiscriminate use of streptomycin which results in emergence of resistance and this is in agreement with Wannaprasat et al. (2011).

Tetracyclines are commonly used for treatment of animal disease before antibiotic susceptibility test is determined. It is the most commonly used antibiotic in Nigeria and many other third world countries (Baggesen et al. 2000). This may explain the reason for the high resistance this drug recorded in this study. In this study, the high level of resistance to tetracycline recorded is in agreement with the findings of Nde and Logue (2007), who reported similar levels of resistance (76.3%) to tetracycline in Salmonella isolated from broiler in Midwestern, United States. Tetracycline has been one of the most commonly used growth promoters and as a result, resistance to tetracycline should be expected (Jones and Ricke, 2003).

In this study, the frequency of resistance to sulfamethaxazole/trimethoprim (75%) and gentamicin 50% in the Salmonella isolates is similar to the findings of Fashae (2010) and Molbalk et al. (2002). The high level of resistance to these drugs in this study and from isolates from food animal reaffirms the importance of the need to strengthening the collaboration between veterinary and public health sectors on the appropriate methods of detection and reporting of zoonotic food borne pathogens (Adesiji and Fagbami, 2006).

The low rate of Salmonella resistance to ofloxacin, ciprofloxacin and ceftriaxone in...
ethidium bromide. L is 100bp-1kb DNA ladder (molecular marker). Samples 18, 22 and 47 are positive for Sul-3-(dfr/G) resistance gene with bands at 100bp. NC is a no DNA template control

Salmonella strains in poultry infections in the study area is ciprofloxacin, followed by oxofloxacin, levofloxacin, ceftriazone and perfloxacin.

The strA/strB genes were not detected in any of the streptomycin resistant Salmonella strains. Similarly, the ten tetracycline resistant strains were negative for tetA and tetB genes. One of the gentamicin resistant Salmonella strains (S. Pullorum) haboured aac (3)-II gene (Figure 1) while none was positive for aac (3)-IV gene. Three of the 10 sulfamethoxazole/trimethoprim resistant strains harboured both sul-1(dfr/A) (with amplificon size of 150bp, Figure 2) and sul 3 (dfr/G) gene (with amplicon size of 100bp, Figure 3). Two of these strains were Salmonella Gallinarum.

In this study strains of S. Gallinarum and S. Pullorum resistant to streptomycin, gentamicin, tetracycline and sulfamethoxazole/trimethoprim were investigated for the presence of some genes that code for resistance to these agents. Streptomycin and tetracycline resistant strains did not haboured the strA/B nor tetA and tetB genes and this finding is in contrast to those of Chiou and Jones (1995), who reported that all the streptomycin resistance isolate contained both strA and strB genes. Similarly, Pezella et al. (2004) reported that 84% of streptomycin resistance isolate contain strA and strB genes. The resistance of Salmonella to streptomycin resistant genes may be due to efflux and this suggests that resistance may be chromosomally mediated. The findings in this study is in disagreement with Nde and Logue (2007), who reported that tetA genes was detected in more than half of the Salmonella isolates examined in North Dakota, USA. Similarly, Deekshit et al. (2012) reported that tetA and tetB resistant genes occur in Italy. The implication of this finding is that resistance may be chromosomal associated. One gentamicin resistant gene aac (3)-II was detected in the study out of 10 Salmonella isolates tested, while non was resistant to gene aac (3) -IV and his finding disagrees with those of Maynard et al. (2003), who found that 10 Salmonella isolates tested positive for gentamicin resistant gene aac (3)-II while 8 tested positive for aac (3)-IV gene. This suggests that the resistance is both plasmid and chromosomally mediated. Three Salmonella isolates tested carried the sulfamethaxazole/trimethoprim resistant genes sul-1-dfr/A and sul-3-dfr/G, and this finding is in agreement with Deekshit et al. (2012), who in his work reported that all Salmonella isolates carried resistant genes for sul-1-dfr/A and sul-3-dfr/G. The possible reason may be due to modification or replacement of antimicrobial target; the resistance here may be plasmid mediated. From the study, the detection of resistant genes observed in aac (3)-II, sul-1-dfr/A and sul-3-dfr/G as indicated by the bands suggests that resistance was plasmid mediated

CONCLUSION

The following resistance genes was detected: aac (3)-II, sul-1-(dfr/A) and sul-3-(dfr/G). This may be the reason for reoccurring antimicrobial resistance observed in treatment of Salmonella in poultry farms in the study area.

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