Molecular and phenotypic characterization of efflux pump and biofilm in multi-drug resistant non-typhoidal *Salmonella* Serovars isolated from food animals and handlers in Lagos Nigeria

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**Abstract**

**Background:** Multidrug resistance efflux pumps and biofilm formation are mechanisms by which bacteria can evade the actions of many antimicrobials. Antibiotic resistant non-typhoidal *Salmonella* serovars have become widespread causing infections that result in high morbidity and mortality globally. The aim of this study was to evaluate the efflux pump activity and biofilm forming capability of multidrug resistant non-typhoidal *Salmonella* (NTS) serovars isolated from food handlers and animals (cattle, chicken and sheep) in Lagos.

**Methods:** Forty eight NTS serovars were subjected to antibiotic susceptibility testing by the disc diffusion method and phenotypic characterization of biofilm formation was done by tissue culture plate method. Phenotypic evaluation of efflux pump activity was done by the ethidium bromide cartwheel method and genes encoding biofilm formation and efflux pump activity were determined by PCR.

**Results:** All 48 *Salmonella* isolates displayed resistance to one or more classes of test antibiotics with 100% resistance to amoxicillin-clavulanic acid. Phenotypically, 28 (58.3%) of the isolates exhibited efflux pump activity. However, genotypically, 7 (14.6%) of the isolates harboured *acrA*, *acrB* and *tolC*, 8 (16.7%) harboured *acrA*, *acrD* and *tolC* while 33 (68.8%) possessed *acrA*, *acrB*, *acrD* and *tolC*. All (100%) the isolates phenotypically had the ability to form biofilm with 23 (47.9%), 24 (50.0%), 1 (2.1%) categorized as strong (SBF), moderate (MBF) and weak (WBF) biofilm formers respectively but *csgA* gene was detected in only 23 (47.9%) of them. Antibiotic resistance frequency was significant (*p* < 0.05) in SBF and MBF and efflux pump activity was detected in 6, 21, and 1 SBF, MBF and WBF respectively.

**Conclusion:** These data suggest that *Salmonella* serovars isolated from different food animals and humans possess active efflux pumps and biofilm forming potential which has an interplay in antibiotic resistance. There is need for prudent use of antibiotics in veterinary medicine and scrupulous hygiene practice to prevent the transmission of multidrug resistant *Salmonella* species within the food chain.

**Keywords:** Efflux pump, Biofilm, Antibiotics, Multidrug resistance, *Salmonella*

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Background

Salmonella are motile rod-shaped (bacilli) Gram-negative bacteria of the family Enterobacteriaceae. Nontyphoidal salmonella serovars mostly cause gastroenteritis, bacteremia, and focal infection. Ingestion of contaminated animal products, such as poultry, pork, and other meats is a major route of transmission in humans. Direct contact is also a potential route of transmission in animals such as chicks, ducklings and other animals that may also transmit the bacterium to humans [1].

Antimicrobial resistance remains a global public health concern threatening the effectiveness of antibacterial therapy. Different variants of bacterial pathogens isolated globally have now become multidrug resistant. Antibacterial resistance occurs by numerous mechanisms including enzymatic inactivation, modification of drugs, drug target alteration or protection, and efflux of drugs through efflux pumps. Antibiotic resistance has been seen in both typhoidal and non-typhoidal serovars [2]. Food animals and handlers contribute largely to the spread of zoonotic multidrug resistant non-typhoidal Salmonella. Efflux generally involves transportation of a substance out of the cell. Efflux pumps play an essential role in the physiology of bacteria by mediating the entry and extrusion of essential nutrients, metabolic waste and xenobiotics. Bacterial efflux systems generally fall into five classes, the major facilitator (MF) superfamily, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family and the multidrug and toxic compound extrusion (MATE) family [3]. Salmonella has at least one MDR pump from each family with the exception of the SMR family of efflux pumps, all of the identified MDR efflux systems also exist in E. coli with the exception of MdsABC (mds-multidrug transporter for Salmonella) which is unique to Salmonella [4]. The best characterised of the RND pumps in Salmonella is AcrB and its tripartite complex AcrAB-ToIC which has many different substrates making this efflux pump (and other RND homologues) a key mediator of multidrug resistance in Gram negative bacteria including many Enterobacteriaceae [5]. Another recently described family of transport protein is the Proteobacterial antimicrobial compound efflux (PACE) systems that is said to be considered across many Gram-negative pathogens including, Klebsiella, Burkholderia, Salmonella, Pseudomonas and Enterobacter species. They have been shown to mediate resistance to several antimicrobials including chlorhexidine, dequalinium, acriflavine, benzalkonium and proflavine [6]. Biofilm formation contributes largely to the resistance of bacteria to different classes of antimicrobials. They can act synergistically with efflux pumps leading to elevated levels of clinical significance. Salmonella biofilms are encased in a matrix largely composed of two major components; curli and cellulose. They are involved in many functions including adhesion, cell aggregation, environmental persistence and biofilm formation [7]. This study was aimed at evaluating the antibiotic resistance profile of NTS serovars as well as their biofilm forming and efflux pump activity potentials.

Methods

Study design and bacterial strains

This descriptive study was conducted in Lagos southwestern Nigeria. Forty eight NTS serovars isolated from food animals comprising 28 isolates from chicken, 3 from cattle, 9 from sheep and 8 from apparently healthy food handlers as previously reported [8].

Antibiotic susceptibility testing

Antibiotic susceptibility testing (AST) was done according to the guidelines of the European committee on antimicrobial susceptibility testing [9] using the disc diffusion method. A loop full of a 24 h brain heart infusion broth culture of isolates were streaked on nutrient agar plate incubated for 24 h at 37 °C. One or two colonies were picked and emulsified in 5 mL of normal saline and adjusted to 0.5 McFarland standard. Using a sterile swab stick, bacterial suspensions were applied to the surface of Muller-Hinton agar (Oxoid, Basingstoke, UK) after which test antibiotic discs were applied and

Table 1 List of Primers Used in Targeting Genes Encoding Efflux Pump and Biofilm Forming Ability

| Primer     | Sequence (5'-3')                     | Product Size (bp) | Reference |
|------------|--------------------------------------|-------------------|-----------|
| acrA-FW    | CTCTCAAGCAGCCTAGGCCCTAA              | 106               | [14]      |
| acrA-RV    | AAGTCAAAAAACGCTCCTGCA                |                   |           |
| acrB-FW    | GGTCGATTCCCGTGTTCTCCGTTA            | 104               | [14]      |
| acrB-RV    | ATGCAGTTTACTCCAGGTTA                |                   |           |
| acrD-FW    | AATTGTGCAGGAACGGCTG                 | 100               | [15]      |
| acrD-RV    | GCTACAGGGCCCATGAA                   |                   |           |
| tolC-FW    | AAGGCCAAAAGCAGCAACCT                | 100               | [14]      |
| tolC-RV    | GATGGTACCTACCCGACTCTG               |                   |           |
| csgA-FW    | GCAATCGATTCTCCGTTA                 | 418               | [16]      |
| csgA-RV    | GATGGACGGTGCCTGTTGTA               |                   |           |
incubated at 37 °C for 24 h. Zones of inhibition were measured and interpreted as Resistance (R), Intermediate (I) and Sensitive (S) with break points of 22–19 for cef-tazidime (30 μg), 19–19 for cefuroxime (30 μg), 17–14 for gentamicin (10 μg), 17–17 for cefixime (5 μg), 24–22 for ofloxacin (5 μg), 19–19 for amoxicillin , clavulanic acid (30 μg), 11–11 for nitrofurantoin (300 μg), 25–22 for cip-rofloxacin (5 μg), 15–11 for tetracycline (30 μg), 19–15 for nalidixic acid (30 μg) and 22–17 for Imipenem (30 μg) (range implies the values between sensitivity ≤ and resistance >). AST was done in duplicates and Escherichia coli ATCC 25922 was used as quality control organism.

### Table 2 Percentage Susceptibility of Isolates to Various Classes of Antibiotics

| Class of Antibiotics | Antibiotics          | Sensitive (%) | Resistant (%) |
|----------------------|----------------------|---------------|---------------|
| Beta-lactam          | Amoxicillin-clavulanic acid | 0             | 100           |
|                      | Ceftazidime          | 6.2           | 93.8          |
|                      | Cefuroxime           | 6.2           | 93.8          |
|                      | Cefixime             | 72.9          | 27.1          |
| Carbapenem           | Imipenem             | 100           | 0             |
| Aminoglycoside       | Gentamicin           | 52.1          | 47.9          |
| Quinolone            | Ciprofloxacin        | 60.4          | 39.6          |
|                      | Ofloxacin            | 68.7          | 31.3          |
|                      | Nalidixic acid       | 58.3          | 41.7          |
| Tetracycline         | Tetracycline         | 31.2          | 68.8          |
| Others               | Nitrofurantoin       | 100           | 0             |

Phenotypic characterization of biofilm formation

**Tissue culture plate method**

Biofilm formation was evaluated by the tissue plate method according to Christensen et al. [10] and Cavant et al. [11] with slight modifications. Isolates were inoculated into brain heart infusion broth (Oxoid, Basingstoke, UK) supplemented with 2% of sucrose and incubated for 18 h at 37 °C. A one in 100 dilution of the culture was made with fresh sterile brain heart infusion broth and 0.2 mL was dispensed into individual wells of a 96 well tissue culture plate. Sterile broth served as negative control and *Salmonella Typhimurium* 14028 was inoculated into separate wells as positive control. Incubation was done at 37 °C for 24 h. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 mL of phosphate buffer saline (PBS pH 7.2) to remove free-floating planktonic bacteria. The plates were then stained with crystal violet (0.1% w/v) and allowed to stay for 45 min. Excess stain was rinsed off by washing with deionized water thrice and plates were allowed to air dry. Crystal violet incorporated by the adherent cells was solubilized by adding 200 μL of 33% glacial acetic acid (Merck, Darmstadt, Germany). The optical density (OD) of each well was determined with an Emax® Plus Microplate Reader (Molecular Devices San Jose, CA) at wave length 570 nm. The experiment was performed in triplicates and repeated three times. Absorbance was calculated by subtracting the OD<sub>570</sub> of control from that of the assays OD<sub>570</sub> with mean value determined for each isolate. Data obtained was used to classify OD<sub>570</sub> values < 0.120 as weak biofilm formers, values between 0.120–0.240 as moderate biofilm formers, and > 0.240 as strong biofilm formers.

![Efflux pump activity of *Salmonella* isolates determined by the ethidium bromide cartwheel method. Isolates A, B, C, D, E, F, G, H are positive for efflux pump activity as they did not fluoresce under UV light. Isolate I lacks efflux pump activity and fluoresced because of ethidium bromide retention.](image-url)
Determination of efflux pump activity by Ethidium bromide cartwheel method

The ethidium bromide cartwheel method according to Martins et al. [12] was used in evaluating efflux pump activity in isolates. Approximately $10^6$ cells per mL of Salmonella isolates were streaked on Muller-Hinton agar plates containing 0 mg/L, 0.5 mg/L, 1 mg/L, 1.5 mg/L and 2 mg/L concentrations of EtBr and incubated at 37 °C for 24 h. After incubation, the plates were examined under UV light. Fluorescence of isolates at different concentrations of EtBr were noted. Isolates without fluorescence indicated active efflux pump activity while those that fluoresced lacked efflux pump activity.

Detection of efflux pump and biofilm encoding genes

Genomic DNA of isolates was extracted according to the method of Kpoda et al. [13] Four efflux pump encoding genes (acrA, acrB, acrD toIC) and one biofilm formation encoding gene (csgA) were assayed for by monoplex PCR targeting specific primers listed in Table 1. A 20 μL PCR reaction was used which contained 10.8 μL nuclease free water, 0.6 μL forward primer, 0.6 μL reverse primer, 4 μL DNA template and 4 μL of 5X PCR Master Mix (7.5 mM MgCl₂, 1 Mm dNTPs, 0.4 M Tris-HCl, 0.1 M (NH₄)_2SO₄, 0.1% Tween-20, FIRE Pol DNA Polymerase) (Solis BioDyne Estonia). PCR was carried out in an Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany) with PCR programming conditions of initial DNA denaturation at 95 °C for 5 min,
followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for csgA, 56 °C for acrA, 54 °C for tolC and 55 °C for acrB and 51 °C for acrD for 30 s and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. PCR products were electrophoresed at 100 V for 1 h in 2% agarose gel stained with ethidium bromide and visualized under ultraviolet transilluminator (Cleaver Scientific Ltd). A 100 bp DNA ladder (Solis Biodyne, Estonia) was used as a molecular weight marker.

Data analysis
Graphics and data analysis were performed by Microsoft Excel (Microsoft Cooperation, 2013 USA) and GraphPad Prism version 8.0.2 (GraphPad Software Inc. USA). One way ANOVA test was used to determined association between variables. Statistical significance was considered for \( p < 0.05 \).

Results
All 48 (100%) Salmonella isolates were susceptible to nitrofurantoin and imipenem. They all (100%) were however, resistant to amoxicillin-clavulanic acid. Forty five (93.8%) of the isolates were resistant to ceftazidime and cefuroxime, 13 (27.1%) were resistant to cefixime, 23 (47.9%) were resistant to gentamicin, 15 (31.3%) were resistant to ofloxacin, 19 (39.6%) were resistant to ciprofloxacin, 33 (68.8%) were resistant to tetracycline and 20 (41.7%) were resistant to nalidixic acid as shown in Table 2. Twenty five (52.1%) of the isolates were multidrug resistant (MDR).

All Salmonella isolates were biofilm formers and 23 (47.9%), 24 (50.0%) and 1 (2.1%) of the isolates were strong (SBF), moderate (MBF) and weak (WBF) biofilm formers respectively. Also 28 (58.3%) of the isolates phenotypically displayed efflux pump activity as they did not fluoresce under UV light since they did not retain ethidium bromide within their cells as shown in Fig. 1. Of the 25 MDR isolates 7 were SBF and 18 were MBF while the WBF was not MDR. Furthermore, efflux pump activity was detected in 6, 21, and 1 SBF, MBF and WBF respectively as shown in Fig. 2. It was also observed that antibiotic resistance frequency was significant (\( p < 0.05 \)) in SBF and MBF as shown in Fig. 3. Although all the isolates had the ability to form biofilm, csgA gene was only detected in 23 (47.9%) of the isolates as shown in Fig. 4.

\[ \text{acrA, acrB and tolC were detected in } 7 \text{ (14.6%) of the isolates, acrA, acrD and tolC were detected in } 8 \text{ (16.7%) of the isolates while } 33 \text{ (68.8%) possessed all four genes acrA, acrB, acrD and tolC. Although majority of isolates from chicken, sheep and human harboured all four efflux pump genes, some did not phenotypically exhibit efflux pump activity as shown in Table 3.} \]

Discussion
In this study all Salmonella isolates were sensitive to imipenem and nitrofurantoin indicating a low pressure on the use of these antibiotics. This is in line with the study of Akinyemi et al. [17] who reported a 100% sensitivity of Salmonella spp. isolated from different sources in Nigeria to imipenem. Similarly, Albert et al. [18] in their findings reported a total sensitivity of NTS isolated from blood stream infection in Kuwait to imipenem. However, antibiotic resistance to various classes of antibiotics was recorded in this study with 52.1% of MDR isolates. Salmonella serovars showed high resistance to third generation cephalosporins where 93.8% were resistant to ceftazidime and cefuroxime and 27.1% were resistant to cefixime. This is in line with the report of

![Fig. 4](image-url)

Fig. 4 Agarose gel image of PCR products showing bands for csgA (418 bp). Lane M: Molecular maker, Lane 1, 5, 6, 9, 11, 12, 15, 18, 21, 22, 23, 27, 28, 29, 30, 31, 32, 34, 35 and 37 are positive for csgA.
| S/N | Code | Source | Serovar | Biofilm Potential | Efflux Pump Activity | Efflux Pump Genotype |
|-----|------|--------|---------|-------------------|----------------------|---------------------|
| 1   | A1   | Chicken| S. Ebrie | Moderate          | Positive             | acrA, acrB, tolC    |
| 2   | A2   | Chicken| S. Budapest | Moderate       | Negative             | acrA, acrD, tolC    |
| 3   | A3   | Chicken| S. Muenster | Moderate        | Positive             | acrA, acrB, tolC    |
| 4   | A4   | Chicken| S. Dabou  | Strong            | Positive             | acrA, acrB, acrD, tolC |
| 5   | A5   | Chicken| S. Budapest | Strong          | Positive             | acrA, acrB, acrD, tolC |
| 6   | A6   | Chicken| S. Tennyson | Moderate        | Positive             | acrA, acrB, acrD, tolC |
| 7   | A7   | Chicken| S. Budapest | Moderate        | Positive             | acrA, acrB, acrD, tolC |
| 8   | A8   | Chicken| S. Brandenburg | Moderate   | Positive             | acrA, acrB, acrD, tolC |
| 9   | A9   | Chicken| S. Anecho | Strong           | Positive             | acrA, acrB, tolC    |
| 10  | A10  | Chicken| S. Minna  | Moderate          | Positive             | acrA, acrB, acrD, tolC |
| 11  | A11  | Chicken| S. Budapest | Moderate        | Negative             | acrA, acrD, tolC    |
| 12  | A12  | Chicken| S. Budapest | Moderate        | Positive             | acrA, acrB, acrD, tolC |
| 13  | A13  | Chicken| S. Budapest | Strong          | Negative             | acrA, acrB, acrD, tolC |
| 14  | A14  | Chicken| S. Budapest | Moderate        | Positive             | acrA, acrB, acrD, tolC |
| 15  | A15  | Chicken| S. Budapest | Strong          | Negative             | acrA, acrD, tolC    |
| 16  | A16  | Chicken| S. Budapest | Moderate        | Positive             | acrA, acrD, tolC    |
| 17  | A17  | Chicken| S. Agodi  | Strong           | Negative             | acrA, acrB, acrD, tolC |
| 18  | A18  | Chicken| S. Budapest | Moderate        | Positive             | acrA, acrB, acrD, tolC |
| 19  | A19  | Chicken| S. Essen   | Strong           | Negative             | acrA, acrB, acrD, tolC |
| 20  | A20  | Chicken| S. Budapest | Moderate        | Positive             | acrA, acrB, acrD, tolC |
| 21  | A21  | Chicken| S. Anecho  | Strong           | Positive             | acrA, acrB, acrD, tolC |
| 22  | A22  | Chicken| S. Agodi   | Strong           | Negative             | acrA, acrD, tolC    |
| 23  | A23  | Chicken| S. Kaapstad | Moderate       | Positive             | acrA, acrB, acrD, tolC |
| 24  | A24  | Chicken| S. Anecho  | Strong           | Negative             | acrA, acrB, acrD, tolC |
| 25  | A25  | Chicken| S. Ealing  | Strong           | Negative             | acrA, acrB, acrD, tolC |
| 26  | A26  | Chicken| S. Wichita | Moderate        | Negative             | acrA, acrB, acrD, tolC |
| 27  | A27  | Chicken| S. Budapest | Moderate        | Positive             | acrA, acrB, acrD, tolC |
| 28  | A28  | Chicken| S. Budapest | Strong          | Negative             | acrA, acrB, acrD, tolC |
| 29  | S1   | Sheep  | S. Chomedey | Strong         | Positive             | acrA, acrB, acrD, tolC |
| 30  | S2   | Sheep  | S. Dahra   | Moderate        | Positive             | acrA, acrB, acrD, tolC |
| 31  | S3   | Sheep  | S. Yovokome| Moderate        | Positive             | acrA, acrB, acrD, tolC |
| 32  | S4   | Sheep  | S. Sculcoates | Strong      | Positive             | acrA, acrB, tolC    |
| 33  | S5   | Sheep  | S. Berlin  | Strong           | Negative             | acrA, acrB, acrD, tolC |
| 34  | S6   | Sheep  | S. Essen   | Strong           | Negative             | acrA, acrB, acrD, tolC |
| 35  | S7   | Sheep  | S. Livingstone | Strong     | Negative             | acrA, acrB, acrD, tolC |
| 36  | S8   | Sheep  | S. Mura    | Moderate        | Positive             | acrA, acrB, acrD, tolC |
| 37  | S9   | Sheep  | S. Orion   | Strong           | Negative             | acrA, acrB, acrD, tolC |
| 38  | H1   | Human   | S. Typhimurium | Moderate  | Positive             | acrA, acrB, acrD, tolC |
| 39  | H2   | Human   | S. Portland | Moderate        | Positive             | acrA, acrB, acrD, tolC |
| 40  | H3   | Human   | S. Limete  | Strong           | Negative             | acrA, acrB, tolC    |
| 41  | H4   | Human   | S. Takoradi | Weak           | Positive             | acrA, acrB, acrD, tolC |
| 42  | H5   | Human   | S. Chagoua | Strong           | Negative             | acrA, acrB, acrD, tolC |
| 43  | H6   | Human   | S. Huettwillen | Strong    | Negative             | acrA, acrB, acrD, tolC |
| 44  | H7   | Human   | S. Paratyphi C | Strong   | Negative             | acrA, acrB, acrD, tolC |
Musa et al. [19] who reported multiple resistance patterns of Salmonella species isolated from human stool samples and raw meat to cefuroxime, cefazidime and ceftriaxone in Niger state Nigeria. In a previous study, Akinyemi et al. [20] in Lagos Nigeria also reported increase in Salmonella spp. resistance to third generation cephalosporin. Resistance of NTS serovars to quinolones in this study was also common. Twenty (41.7%), 15 (31.3%) and 19 (39.6%) Salmonella serovars were resistant to nalidixic acid, ofloxacin and ciprofloxacin respectively. Quinolones most especially ciprofloxacin remains a drug of choice for the treatment of Salmonella. However the wide spread resistance to this antibiotic is worrisome. Katiyo et al. [21] in a study between 2004 and 2015 of NTS bacteremia in England revealed a high resistance to ceftriaxone in this pathogen in extruding extraneous materials including antibiotics and in maintaining viability.

**Table 3** Source of Salmonella Serovars, their Biofilm Forming Potential, Efflux Pump Activity and Efflux Pump Genotype (Continued)

| S/N | Code | *Source* | Serovar | Biofilm Potential | Efflux Pump Activity | Efflux Pump Genotype |
|-----|------|----------|--------|-------------------|----------------------|----------------------|
| 45  |     | Human    | S. Mowanjum | Moderate           | Positive             | acrA, acrB, acrD, tolC |
| 46  | CO1  | Cow      | S. Budapest  | Moderate           | Positive             | acrA, acrD, tolC     |
| 47  | CO2  | Cow      | (43.g, zrg,enz) II | Moderate          | Positive             | acrA, acrB, tolC     |
| 48  | CO3  | Cow      | S. Sommer  | Strong             | Negative             | acrA, acrB, tolC     |

*Source of Salmonella isolates and serotypes previously reported in Ajayi et al. [8]*

Conclusion

In this study several Salmonella serovars isolated from food animals and food handlers were multidrug resistant which would have been mediated by efflux pump activity and biofilm formation potentials which they possessed. Beyond mediating antibiotic resistance, biofilm formation by Salmonella spp. also enable them to be persistent in food processing environments hence promoting their transmission and colonization of multiple hosts. Therefore, food, personal and environmental hygiene is imperative with constant epidemiological surveillance to track and control the transmission of the pathogen within the food chain.

**Abbreviations**

EBr: Ethidium bromide; MDR: Multidrug resistant; MBF: Moderate biofilm former; MDR: Resistance nodulation division; SBF: Strong biofilm former; OD: Optical density; WBF: Weak biofilm former

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Not applicable.

**Authors’ contributions**

Olubisose ET and Ajayi contributed to data collection and preparation of draft; Smith SI and Adeleye AI contributed to conceptualization of study, supervision and correction of draft. The authors read and approved the final manuscript.

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