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Effects of Plasmid Curing on Multi-Drug-Resistant *Salmonella* from Poultry Litter in Nasarawa Town, Nasarawa State, Nigeria

Yakubu Aliyu[1]*, Chrinius Hammuel[2], Ajang Abubakar Yakubu[3], and Justina Adegba[1]

[1]Department of Applied Biology/Microbiology, School of Applied Sciences, Federal Polytechnic, Nasarawa, Nigeria
[2]Department of Microbiology, Faculty of Biosciences, Federal University Wukari, Tabara State, Nigeria
[3]Department of Microbiology, Faculty of Natural and Applied Sciences, University of Jos, Plateau State, Nigeria
*Correspondence author: aleeyaqub29@gmail.com

Abstract

The emergence of antibiotic resistance in bacteria, often mediated by chromosomal or plasmid-borne genes, poses a significant public health concern. This study investigated the impact of plasmid curing on multidrug-resistant Salmonella *isolates recovered from poultry litter samples in Nasarawa town, Nasarawa State, Nigeria. A total of 96 poultry litter samples were processed. Using standard microbiological protocols,* Salmonella *species were isolated, characterised, tested for antibiotic susceptibility, and subjected to plasmid curing. Twenty-three* Salmonella *isolates were screened against ten commonly used antibiotics using the agar disc diffusion technique. Resistance levels of 56.5%, 43.5%, 34.9%, 39.2%, and 22% were observed for Augmentin, Sparfloxacin, Gentamicin, Co-trimoxazole, and Streptomycin, respectively, indicating that they are multidrug-resistant Salmonella strains. Acridine orange was employed as the curing agent. Notably, all five strains initially resistant to streptomycin became susceptible post-curing. Similarly, all 13 strains resistant to augmentin and 4 out ofthe 9 that were resistant to co-trimoxazole lost resistance after plasmid curing. Additionally, 6 out of 10 sparfloxacin-resistant strains became susceptible post-treatment. These findings suggest that plasmid-mediated mechanisms contribute significantly to multidrug resistance in poultry-derived* Salmonella. *Further molecular studies are recommended to characterize resistance plasmids and inform policies on prudent antibiotic use in animal husbandry.*

Keywords: Plasmid Curing, Multi-drug Resistance, Salmonella, Poultry Litter

INTRODUCTION

Plasmids are autonomously replicating, circular DNA molecules distinct from chromosomal DNA and commonly found in bacterial cells. Although they constitute a small proportion of the total genetic content—typically around 2%—they often carry genes that confer a selective advantage, including antibiotic resistance and metal tolerance (Mayer, 2019; Adeyemo &Onilude, 2019). These mobile genetic elements are key drivers of horizontal gene transfer, facilitating the rapid dissemination of resistance traits within and across bacterial populations (Dasmeh *et al.*, 2015).

Plasmid curing refers to the intentional removal of plasmids from bacterial cells, often using chemical or physical agents (Ozdemir, 2019). This technique is employed to determine the genetic basis of resistance, particularly whether a trait is plasmid-encoded or chromosomally integrated (Ozdemir, 2019). Compounds like acridine orange can inhibit plasmid replication without affecting chromosomal DNA, thereby selectively eliminating plasmids from bacterial cells (Wadud *et al.*, 2013). While the therapeutic application of plasmid-curing agents remains limited, their experimental use provides insights into resistance mechanisms and opens potential avenues for managing antimicrobial resistance (AMR), especially in agricultural and clinical settings (Ijabani *et al.*, 2022).

Multidrug resistance (MDR) in bacteria is a growing concern globally (Wemedo *et al.*, 2022). It can result from the presence of resistance genes on R-plasmids or transposons, overexpression of multidrug efflux pumps, or enzymatic drug deactivation (Bennett, 2018). In the case of zoonotic pathogens such as *Salmonella*, MDR strains are particularly alarming due to their prevalence in food-producing animals and potential transmission to humans via the food chain (Helal *et al.*, 2019).

Salmonella is recognised as a major foodborne pathogen and a common contaminant in poultry environments (Ibrahim *et al.*, 2019). The bacterium has been frequently isolated from poultry litter, a byproduct widely used as

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organic fertilizer due to its rich nutrient profile (Wegener *et al.*, 2021; Aires, 2019). Despite its agricultural benefits, poultry litter is also a reservoir for antibiotic-resistant bacteria and resistance genes, posing environmental and public health risks (Peng *et al.*, 2017).

The rise in *Salmonella enterica* serovar Enteritidis infections, linked to both increased virulence and resistance, underscores the importance of monitoring MDR strains in poultry systems (Kid *et al.*, 2019). As highlighted by Chander *et al.* (2017), antibiotic resistance is one of the most pressing health threats of our time, with the potential to reverse decades of medical progress.

This study, therefore, aimed to evaluate the effectiveness of plasmid curing in reversing antibiotic resistance among *Salmonella* isolates obtained from poultry litter in Nasarawa town, thereby providing insights into the molecular basis of resistance in the local agricultural setting.

MATERIALS AND METHODS

Study Area

This study was conducted in Nasarawa town, Nasarawa State, Nigeria. The town spans approximately 570.4 square kilometres and had a population of 189,835 according to the 2006 census (Abdulkarim *et al.*, 2016). Nasarawa town is located about 105 kilometres from Abuja, the Federal Capital Territory, 37 kilometres from Keffi, and 165 kilometres from Lafia, the capital of Nasarawa State. The geographical coordinates of the study location are approximately 8°21'58" N latitude and 7°5'58" E longitude (Abdulkarim *et al.*, 2016).

Sample Size

The number of poultry litter samples collected was determined using an estimated prevalence rate of 6.7% for *Salmonella* in poultry litter, as reported by Marik *et al.* (2022). The sample size was calculated using a standard formula for sample size determination in prevalence studies, as outlined by Naing *et al.* (2006), which accounts for the expected prevalence, desired precision, and confidence level. Based on this calculation, a total of ninety-six (96) poultry litter samples were collected for the study.

$$n = \frac{z^2 \mathrm{p}(1-\mathrm{p})}{d^2}$$

where; n = sample size

P = Prevalence from a previous study

= 6.7% = 0.067

Z = Standard normal distribution at 95% confidence interval = 1.96

D = Absolute desired precision at 5% = 0.05

Therefore, $n = \frac{z^2 \mathrm{p}(1-\mathrm{p})}{d^2}$

$$n = \frac{1.96^2 \mathrm{x}\ 0.067(1-0.067)}{0.05^2}$$

$$n = \frac{3.8416 \mathrm{x}\ 0.067(1-0.933)}{0.0025}$$

$$n = \frac{0.24014}{0.0025}$$

n = 96.01 ~ 96

$$n = 96$$

Sample Collection

A set of 96 poultry litter specimens was methodically gathered from four distinct commercial poultry operations located in Nasarawa town. Written informed consent was secured from all participating farm managers before initiating sample collection. Employing sterile, screw-top containers, samples were obtained using aseptic protocols and promptly conveyed under appropriate conditions to the Microbiology Laboratory within the Applied Biology/Microbiology Department, Federal Polytechnic, Nasarawa, for subsequent microbial analysis.

Isolation of *Salmonella* Species

Salmonella isolation adhered strictly to the World Organisation for Animal Health (OIE, 2012) guidelines. One (1) gram of each litter specimen was homogenized in 9 mL of buffered peptone water (BPW) for primary enrichment, followed by incubation at 37 °C for 24 hours. Next, a 1 mL aliquot from this pre-enriched suspension was inoculated into 10 mL of selenite cysteine broth for selective enrichment, with a further 24-hour incubation at 37 °C. Following enrichment, an inoculating loop was used to streak cultures onto Salmonella-Shigella Agar (SSA) plates. After incubation at 37 °C for 24 hours, plates were inspected for presumptive *Salmonella* colonies, identified by their translucent, pinkish-red morphology often featuring dark central points. Suspect colonies were purified by sub-culturing onto fresh SSA

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plates. Resulting pure isolates were maintained on nutrient agar slants for subsequent biochemical characterization and antibiotic susceptibility profiling.

Biochemical Identification and Characterisation of *Salmonella* Isolates

Presumptive Salmonella identification relied on Gram staining reactions and a standardized battery of biochemical assays: Triple Sugar Iron agar, Sulphide-Indole-Motility (SIM) medium, Methyl Red-Voges Proskauer (MR-VP) tests, citrate utilization, and urease activity. TSI agar slants, prepared per manufacturer's instructions, were inoculated using stab-and-streak techniques and incubated at 37 °C for 24–48 hours. *Salmonella* typically produces an alkaline (red) slant and acid (yellow) butt, often with gas formation and a darkened base portion indicating H_2S generation (Cheesbrough, 2010).

SIM medium assessed motility, H_2S production, and indole formation. Tubes were stabbed with pure cultures and incubated at 37 °C for 24 hours. Diffuse growth radiating from the stab line signified motility, while a black precipitate confirmed H_2S production. Kovac's reagent (3 drops) was added for the indole test; a distinct crimson surface ring denoted a positive reaction (Cheesbrough, 2010).

For the MR-VP test, isolates were grown in MR-VP broth (37 °C, 24 hours). The MR test involved adding methyl red indicator to 1 mL of culture; an immediate red color signified a positive (acidic) result. The VP test used the remaining broth, treated with 5 drops each of 40% KOH and 5% α-naphthol solution. Tubes were gently agitated and observed for up to one hour; development of a red surface ring indicated a positive VP result. *Salmonella* characteristically exhibits MR-positive and VP-negative reactions (Cheesbrough, 2010). Citrate utilisation was evaluated on Simmons' citrate agar slants. After streaking the slant surface with a sterile needle carrying the test isolate and incubating (37 °C, 24 hours), a distinct blue color change signified positive citrate utilisation, a trait common to *Salmonella* (Cheesbrough, 2010).

Urease activity was determined by streaking isolates onto urea agar slants and incubating (37 °C, 24 hours. A positive result, indicated by a magenta-pink color throughout the medium, is atypical for *Salmonella*, which generally yields a negative test (unchanged yellow medium) (Cheesbrough, 2010).

Antibiotic Susceptibility Testing of Confirmed *Salmonella* Isolates

Inoculum Preparation and Standardisation

Inocula for susceptibility testing were prepared by emulsifying pure *Salmonella* colonies from nutrient agar in sterile physiological saline (0.85% NaCl). The turbidity of each suspension was meticulously calibrated to the 0.5 McFarland standard (approximating 1.5×10^8 CFU/mL) by adding colonies or saline, ensuring uniform bacterial density essential for reliable sensitivity testing (Cheesbrough, 2010).

Antibiotic Susceptibility Assay

Antimicrobial susceptibility of confirmed *Salmonella* isolates was determined using the standardized Kirby-Bauer disc diffusion method on Mueller-Hinton agar, following Clinical and Laboratory Standards Institute protocols (CLSI, 2017). Ten antimicrobial agents were evaluated: amoxicillin (30 µg), chloramphenicol (30 µg), ciprofloxacin (30 µg), gentamicin (30 µg), streptomycin (30 µg), sparfloxacin (10 µg), pefloxacin (30 µg), amoxicillin-clavulanate (10 µg), ofloxacin (10 µg), and trimethoprim-sulfamethoxazole (30 µg). Standardised suspensions (0.5 mL) were uniformly lawn-cultured onto Mueller-Hinton agar plates using sterile swabs. Antibiotic discs were aseptically applied to the inoculated surfaces. Plates were incubated at 37°C for 24 hours. Inhibition zone diameters surrounding each disc were measured precisely using a calibrated ruler. Results were categorized as susceptible, intermediate, or resistant based on established CLSI interpretive criteria (CLSI, 2017). Isolates demonstrating resistance to three or more distinct antimicrobial classes were designated as multidrug-resistant (MDR), per Intrakamhaeng and Komutarin (2012).

Plasmid Curing and Post-curing Antibiotic Sensitivity Evaluation

Plasmid elimination was attempted using an acridine orange-based method adapted from Raghada *et al*. (2013). Cultures (10 mL) were exposed to peptone water supplemented with 20 µL of acridine orange solution and incubated at 37°C for 24 hours. Subsequently, 1 mL aliquots from each treated culture were spread onto nutrient agar plates and incubated again (37°C, 24 hours) to recover potentially cured derivatives.

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Candidate cured colonies were subcultured onto fresh Mueller-Hinton agar (devoid of curing agents). The antibiotic discs corresponding to the isolate's initial resistance profile were reapplied. After incubation (37°C, 24 hours), inhibition zones were remeasured to assess any alteration in susceptibility patterns potentially resulting from plasmid loss.

RESULTS

Seven (7) out of the 24 samples collected from *Oversea* were found to have *Salmonella* giving a prevalence of 29.2%. The percentage occurrence of *Salmonella* in samples collected from Tammah, Mangoro Goma, and Gunki was 16.7, 25.0, and 25.0, respectively. Despite the differences in percentage occurrence, no statistically significant relationship (X^2 = 0.224) was found between the occurrence of *Salmonella* in the poultry droppings and the different areas sampled using chi-square (Table 1).

The results of the antibiotic susceptibility tests of the *Salmonella* isolates obtained are presented in Table 2. Among these isolates, 13 (56.5%) were resistant to Augmentin; 10 (43.5%) were resistant to amoxicillin and sparfloxacin, respectively; and 9 (39.1%) were resistant to co-trimoxazole. However, 9(39.1%) of the isolates were susceptible to streptomycin, 8(34.9%) to gentamicin, and 7(30.4%) to pefloxacin.All of the *Salmonella* isolates are multidrug-resistant strains as they have demonstrated resistance to more than three antibiotic classes.

All of the 10(43.5%), 2(8.7%) 8(34.9%) and 9(39.1%) *Salmonella* isolates that were initially resistant to sparfloxacin, ciprofloxacin, gentamicin, and co-trimoxazole respectively became susceptible to the antibiotics after plasmid curing. However, only 5 (38.5%) out of the 13 isolates that were resistant to augmentin became susceptible to the drug after plasmid curing (Table 3).

Table 1: Occurrence of *Salmonella* in Poultry Litter in Areas sampled

Location	No. Examined	No. Positive	% Occurrence	x^2-Value
Oversea	24	7	29.2%	
Tammah	24	4	16.7%	
Mangoro Goma	24	6	25.0%	
Gunki	24	6	25.0%	0.244
Total	**96**	**23**	**23.9%**	

No. = Number

Table 2: Antibiotic Susceptibility Profile of *Salmonella* from Poultry Litter in Nasarawa Town

		N= 23		
Antibiotic	Conc. (µg)	S (%)	I(%)	R (%)
Amoxicillin	30	3(13.0)	10(43.5)	10(43.5)
Chloramphenicol	30	3(13.0)	16(69.5)	4(17.4)
Ciprofloxacin	30	0(0.00)	21(91.3)	2(8.7)
Gentamicin	30	8(34.9)	7(30.4)	8(34.9)
Streptomycin	30	9(39.1)	9(39.1)	5(22.0)
Sparfloxacin	10	0(0.00)	13(56.5)	10(43.5)
Pefloxacin	30	7(30.4)	12(52.2)	4(17.4)
Augmentin	10	5(21.7)	5(21.7)	13(56.5)
Ofloxacin	10	4(17.4)	15(65.2)	4(17.4)
Co-trimoxazole	30	7(30.4)	7(30.4)	9(39.1)

N = Number, Conc. = Concentration, S = Susceptibility, 1 =Intermediate, R= Resistance

DISCUSSION

This study revealed that 23 out of 96 poultry litter samples (23.9%) were contaminated with *Salmonella* species (Table 1). This prevalence presents a significant public health concern, given the widespread use of poultry litter as organic fertiliser. Although beneficial for agriculture, poultry litter can serve as a vector for the transmission of enteric pathogens such as *Salmonella*, *Escherichia coli*, and *Campylobacter* (Helal *et al.*, 2019; Ijabani *et al.*, 2022). When applied to farmlands, contaminated litter may lead to crop contamination and contribute to outbreaks of foodborne illnesses (Hruby *et al.*, 2018; Chen *et al.*, 2019). Furthermore, runoff from fields treated with such litter can introduce

Salmonella into nearby water bodies (Shepherd *et al.*, 2015; You *et al.*, 2018).Similar findings were reported by Jemilehin *et al.* (2015) and Omoya *et al.* (2016) in Akure, Nigeria, where poultry litter was found to harbour pathogenic bacteria posing considerable health risks.

Table 3: Antibiotic Susceptibility Profile of the Multi-Drug-Resistant Salmonella after Plasmid Curing

Antibiotic	Conc. (µg)	Resistance Before Curing (No. of Isolates)	Resistance After Curing (No. of Isolates)
Sparfloxacin	10	10	None
Ciprofloxacin	30	2	None
Gentamicin	30	8	None
Co-trimoxazole	30	9	None
Augmentin	10	13	5

The 23.9% prevalence in this study is notably higher than the 17.7% and 14.4% reported by Bushen *et al.* (2021) and Waktole *et al.* (2024), respectively, in various regions of Ethiopia. This variation may stem from differences in poultry management practices, environmental conditions, and hygiene standards, all of which influence *Salmonella* contamination rates.

Antibiotic susceptibility testing revealed high resistance rates to augmentin (56.5%) and amoxicillin (43.5%), potentially due to the unregulated and indiscriminate use of these antibiotics in poultry farming. Such practices create selective pressure that facilitates the emergence of resistant strains. The absence of robust antibiotic stewardship or regulatory frameworks likely exacerbates this issue. Furthermore, resistance to one ß-lactam antibiotic often confers cross-resistance to other ß-lactams due to structural and mechanistic similarities. The observed amoxicillin resistance (43.5%) is lower than the 73.3% reported by Samy *et al.* (2022) in Egypt, and significantly lower than the 100% resistance observed by Talukder *et al.* (2021), underscoring geographical disparities in antibiotic usage and resistance dynamics.

Interestingly, 34.9% of isolates were resistant to gentamicin, contrasting with the 26.7% reported by Waktole *et al.* (2024). Since gentamicin is not commonly used in poultry production in Nigeria, this resistance may be attributed to environmental contamination with gentamicin-resistant *Salmonella* strains of human origin. Similarly, resistance levels of 39.1% to co-trimoxazole and 43.5% to sparfloxacin—despite limited or no usage in poultry—suggest the introduction of resistant strains via indirect environmental routes, such as contaminated water or waste.

Crucially, all *Salmonella* isolates exhibited multidrug resistance (MDR), defined as resistance to three or more antibiotic classes (Magiorakos *et al.*, 2012). This aligns with findings by Helmuth and Fashae (2018) in Ibadan, Nigeria. The presence of MDR *Salmonella* in poultry waste is alarming, as such strains can enter the human food chain and cause infections that are difficult to treat, thereby increasing morbidity and mortality risks.

Plasmid curing experiments further demonstrated that resistance to sparfloxacin, co-trimoxazole, gentamicin, and ciprofloxacin was plasmid-mediated. The fact that isolates previously resistant to these antibiotics became susceptible post-curing confirms this mechanism. The use of acridine orange, an intercalating agent, successfully eliminated plasmid-mediated resistance, underscoring its potential utility in managing antibiotic resistance.

These findings emphasize the urgent need for regulatory control over antibiotic use in animal husbandry. The demonstrated effectiveness of plasmid curing supports its potential as a research tool in combating antibiotic resistance. Consistent with earlier reports (Zhang *et al.*, 2012; Barika *et al.*, 2024), the results confirm that acquired (rather than induced) resistance mechanisms were involved. This also validates those certain injectable antibiotics may retain efficacy if their resistance genes are not chromosomally encoded (Guillermo *et al.*, 2012).

CONCLUSION

This study identified a 23.9% prevalence of multidrug-resistant *Salmonella* in poultry droppings, highlighting serious public health risks associated with the use of untreated

poultry litter as fertilizer. The isolates showed resistance to multiple antibiotics, including augmentin, amoxicillin, sparfloxacin, co-trimoxazole, and gentamicin. Significantly, the use of acridine orange successfully reversed resistance, demonstrating that the antibiotic resistance in these strains was plasmid-mediated. These findings underscore the need for proper composting of poultry litter before use in agriculture, as well as public awareness campaigns on the dangers of indiscriminate antibiotic use. Enhanced antibiotic stewardship, along with sound regulatory policies, is essential to prevent further escalation of antibiotic resistance. In the long term, such interventions will be critical in preserving the effectiveness of existing antibiotics and protecting both human and animal health.

CONFLICT OF INTEREST

The authors declare that no conflict of interest exists regarding the publication of this study.

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