Antimicrobial Drug Resistance in Strains of *Salmonella* Isolated from Pig Effluents in Abakaliki, Nigeria

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Abstract: Antimicrobial resistance (AMR) is a current health menace that strikes at the core of infectious disease control, and has potential to affect every aspect of healthcare systems, as well as impact the food chain in terms of the evolution and transmission of antibiotic-resistant bacteria of public health risk. This study investigated antimicrobial drug resistance in strains of *Salmonella* spp. isolated from pig effluents in Abakaliki, Nigeria. Standard microbiology techniques including microscopy, biochemical testing and culture on *Salmonella-Shigella* agar (SSA) was used for the isolation of forty strains of *Salmonella* species from the pig-effluent-samples. Antibiogram studies were conducted by modified disk diffusion technique; and multiple antibiotic resistance index (MARI) was determined. Our results shows that resistance of *Salmonella* spp. to beta-lactam agents was most common, including amoxicillin (97.5%), oxacillin (100%), vancomycin (100%) and penicillin (95%). The *Salmonella* spp. was highly resistant to chloramphenicol (90%); and the strains exhibited multiple resistances to at least 3 antibiotic classes. This preliminary study has shown that *Salmonella* spp. of pig origin is highly resistant to some first- and second-line antibiotics used in clinical medicine. Our next step is to use genomics to characterize the genes responsible for multiple antibiotic resistances in the *Salmonella* spp. Since AMR increasingly threaten human health, it is important to detect and report drug resistance to guide therapy. We recommend alternative approaches that eliminate antibiotic use in livestock.

Keywords: Multidrug Resistance, One Health, Livestock, *Salmonella*

INTRODUCTION

Worldwide increase of *Salmonella* species, particularly *S. typhimurium* has been observed in pig herds since the mid-1990s; the infected pigs shed *Salmonella* through feces during the whole fattening period, and the *Salmonella* bacteria are then transmitted to slaughterhouses from where slaughter lines and carcasses becomes contaminated, thereby posing potential health risk to human population (Sisak *et al*., 2006; Casanova *et al*., 2019). Antibiotic-resistant *Salmonella* spp. remains a (priority) high ranking Gram negative bacteria in the World Health Organization (WHO) priority list of multidrug-resistant (MDR) bacteria that require research and development of new The emergence and transmission of *Salmonella* spp. with potential to resist the antimicrobial onslaughts of antibacterial agents is a cause for concern. The use of antibiotics (WHO, 2017). With the increase in commercial pig farming in Nigeria, there is a simultaneous increase in the use of antibiotics in pig farming coupled with the resultant salmonellosis that may result from the consumption of pigs harbouring antibiotic-resistant *Salmonella* spp. Food-borne diseases caused by *Salmonella* spp. are a serious public health problem in many parts of the world (Casanova *et al*., 2019; Ugwu *et al*., 2019). *Salmonella* infection remains a major public health concern worldwide; and it contributes to the economic burden of both industrialized and underdeveloped countries through the costs associated with surveillance, prevention and treatment of foodborne infections or intoxications caused by these pathogens. antibiotics in agriculture is widespread in Nigeria; and the limited information on drug resistant bacteria from our previous studies shows that antimicrobial resistance (AMR) is a major challenge in Nigeria's healthcare sector (Ejikeugwu *et al*., 2018; 2019; Ugwu *et al*., 2019). Since AMR is a global public health issue with potential to affect the efficacy of available antimicrobial agents, it is important to always be on the lookout for the evolution and transmission of AMR bacteria, particularly in the non-hospital environment (e.g., farms and livestock production facilities) where antibiotic use is usually irrational and not controlled. The surveillance and reporting of AMR and antibiotic usage is a global health priority (Casanova *et al*., 2019; Shaban *et al*., 2014); and the WHO already outlined a priority list of antibiotic-resistant bacteria that includes *Salmonella* spp. since AMR poses a substantial threat to morbidity and mortality globally (WHO, 2017). Whilst the direct use of antimicrobial agents in human health is recognized as a major contributor to antimicrobial resistance in human pathogens, there are circumstances where antimicrobials used in both food-producing and companion animals are important contributing factors (Ugwu *et al*., 2019; Shaban *et al*., 2014). Recent studies have shown that the world is currently facing high rates of AMR; and it is estimated that by 2050, 10 million deaths per year will be attributable to AMR worldwide (Hooban *et al*., 2020). Furthermore, new forms of AMR are emerging and spreading globally, threatening our ability to treat common infectious diseases such as pneumonia. Our previous studies showed that some novel AMR genes and bacteria including MBL- and AmpC-producing Gram negative bacteria are on the increase in the non-hospital environment particularly in abattoir and poultry environments in southeast Nigeria (Ejikeugwu *et al*., 2021, 2020, 2019, 2018); thus signifying the possible contribution of antibiotic use in agriculture and livestock production in the development and transmission of AMR in these milieus. Since humans and animals share many of the same bacteria including *Salmonella* spp. that could be zoonotic in nature, it is important to investigate the susceptibility profiles of these pathogens that can be transmissible between humans and animals. This preliminary study investigated the resistance patterns in strains of *Salmonella* isolated from pigs effluents in Abakaliki, Nigeria – as panacea to update on the susceptibility profile of this important human pathogen, especially in the face of an outbreak caused by the organism.

MATERIALS AND METHODS

Ethics: This research was carried out in line with the World Medical Association (WMA) declaration of Helsinki on the principles for medical research involving animal subjects and identifiable material/data (WMA, 2004). All experiments were approved by the Local Ethics Committee of Ebonyi State University (EBSU), Abakaliki, Nigeria, and were carried out in strict compliance with the WMA guidelines.

Collection of samples: From September 2021 to January 2022, a total of 50 non-duplicate effluent samples were collected from the drinking water, fecal matter and feeding pan of pigs from 10 different pig farms in Abakaliki metropolis of Ebonyi State, Nigeria. The pig effluent samples were inoculated in nutrient broth and transported to the microbiology laboratory of EBSU for further microbiological analysis and isolation of *Salmonella* bacteria.

Culture, isolation and identification of bacteria: Standard microbiology techniques including culture techniques, microscopy, Gram staining, biochemical testing, morphological and bacterial-colonial features on selective and differential culture media including MacConkey agar and Salmonella-Shigella Agar (Oxoid, UK) were aseptically carried out for the selective isolation of strains of *Salmonella* spp. using a previously described technique (Ejikeugwu *et al*., 2018; 2020). All culture plates were incubated at 37^{o}C for 18-24h.

Antibiogram: Antimicrobial susceptibility studies were carried out by the modified Kirby-Bauer disk diffusion method according to the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2017) using single antibiotic disks from Oxoid Limited [Oxoid, UK]. The *Salmonella* spp. test isolates were adjusted to 0.5 McFarland turbidity standards and applied onto Mueller-Hinton (MH) agar plates using sterile swab sticks (Ejikeugwu *et al*., 2019). Single antibiotics disks (as above) were aseptically placed on the swabbed MH agar plates at a distance of 20mm apart using sterile forceps. All susceptibility test plates were incubated at 37°C for 18–24h (Ejikeugwu *et al*., 2019). The zone of inhibition was measured, recorded, and interpreted as susceptible (S) and resistant (R) using standard antibiotic breakpoints as stated by the CLSI (CLSI, 2017).

Determination of multiple antibiotics resistance index (MARI): To determine MARI, the formular: MARI=a/b was used; where **"a"** represents the number of tested antibiotics that the resistant *Salmonella* spp. was resistant to, and **"b"** represents the total number of antibiotics for which the resistant *Salmonella* spp. has been evaluated for (Ejikeugwu *et al*., 2020).

RESULTS

Out of the fifty (50) pig effluent samples obtained from the pig farms, a total of 40 isolates of *Salmonella* spp. were bacteriologically recovered from the various effluent samples analyzed in this present study. Out of these 40 strains of *Salmonella* spp., 15 (30%) strains of *Salmonella* spp. were recovered from the pig's drinking water and 17 (34%) strains of *Salmonella* spp. were isolated from the fecal matter of the pigs. Only 8 (16%) strains of *Salmonella* spp. were isolated from the feeding pans of the pigs (Table 1). *Salmonella* is a bacterium of interest in human population because it is implicated in a number of foodborne and waterborne infections.

The antimicrobial susceptibility and resistant patterns of the strains of *Salmonella* spp. to some commonly used antibiotics is shown in Figure1. This preliminary study on the antimicrobial drug resistance in strains of *Salmonella* spp. isolated from pig effluents showed a very high resistance pattern of the *Salmonella* isolates to some important first- and second-line antibiotics used in clinical medicine globally. The antimicrobial resistance of the *Salmonella* spp. isolates was high to amoxicillin (97.5%) and oxacillin (100%). Very high reduced susceptibility of the *Salmonella* spp. isolates was also recorded against chloramphenicol (90%) and vancomycin (100%). The *Salmonella* spp. isolates also showed significant reduced susceptibility to penicillin (95%), an important first-line beta-lactam agent used for treating commonly encountered bacterial infections (Figure 1). Table 2 shows the results of the multiple antibiotic resistance indexes (MARI) of eight (8) resistant strains of *Salmonella* spp. investigated in this study.

The strains of *Salmonella* spp. isolated in this study were multidrug resistant in nature. It was observed that the MARI's of the *Salmonella* spp. investigated in this study was in the range of 0.2-0.4, with the highest level of multiple resistances recorded against ciprofloxacin (a fluoroquinolone), chloramphenicol (a protein synthesis inhibitor), and amoxicillin and penicillin – which are both beta lactam agents (Table 2). Interestingly, the *Salmonella* spp. had an average MARI of 0.3, thus indicating that the resistant *Salmonella* spp. isolates tested in thus study were resistant to at least three (3) different antibiotics inclusive of two (2) antibiotics from different classes (Table 2).

Table 1. Distribution of strains of *Salmonella* spp. from various pig effluents

Source	No. of (effluent) sample	*Salmonella* spp. n(%)
Drinking water	20	15(30)
Fecal matter	20	17(34)
Feeding pan	10	8(16)
Total	**50**	**40**

Table 2. Result of multiple antibiotics resistance indexes (MARI's) of the *Salmonella* spp.

Isolate Nos.	MARI	Antibiotics
Salm 15	0.2	CIP, OX
Salm 8	0.3	PEN, VAN, CIP
Salm 12	0.4	CIP, CHL, AMX, PEN
Salm 23	0.2	AMX, OX
Salm 9	0.3	PEN, CHL, VAN
Salm 32	0.3	OX, CIP, AMX
Salm 18	0.3	OX, CHL, PEN
Salm 3	0.2	AMX, VAN

Key: Salm – *Salmonella*, CIP- ciprofloxacin, OX-oxacillin, PEN-penicillin, VAN-vancomycin, AMX-amoxicillin, CHL-chloramphenicol

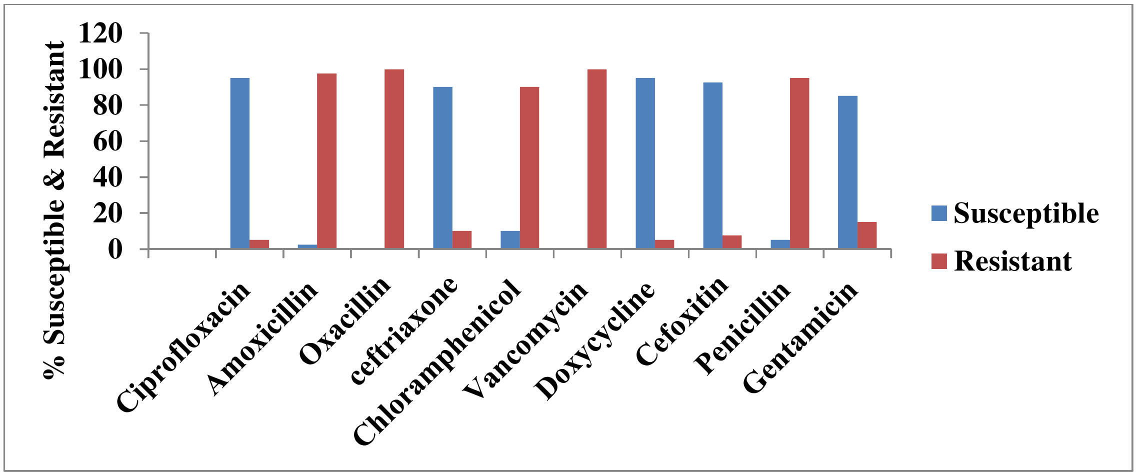

Figure 1. Percentage susceptibility and resistant profiles of *Salmonella* spp.

DISCUSSION

The role of food-producing environments including livestock production facilities and pig farms in the evolution and transmission of antimicrobial resistance (AMR) and multidrug resistant (MDR) bacteria in Nigeria have not yet been fully investigated. This study confirmed a high occurrence rate of antibiotic resistance in the strains of *Salmonella* spp. we isolated from pig effluents in Abakaliki, Nigeria. The *Salmonella* spp. isolates investigated in this study were subjected to antimicrobial susceptibility studies, and we found that the frequency of resistance against the major beta-lactam agents including amoxicillin (97.5%) and penicillin (95%) was very high, and this was also observed with chloramphenicol (90%), a broad-spectrum (non-beta-lactam) agent that inhibits protein synthesis in bacteria, to which the

Salmonella spp. also exhibited a high level These results are comparable with statistics from a previous study conducted in Czech Republic, where high levels of resistance to chloramphenicol (83.0%) and tetracycline (84.7%) was reported amongst *Salmonella* spp. displaying the *ACSSuT* phenotype (Sisak *et al*., 2006). We also found that the *Salmonella* spp. isolates were completely resistant to oxacillin (100%) and vancomycin (100%) – which are important cell wall inhibitors and clinically relevant first-line antibiotics used for treating bacterial infections caused by both Gram positive and Gram negative bacteria. The prevalence of antimicrobial resistance among the *Salmonella* spp. isolates in this study was found to be similar to that reported in China (Jiang *et al*., 2019) and Czech Republic (Sisak *et al*., 2006) where *Salmonella* spp. isolated from pigs were found to be resistant to some commonly used first-line antibiotics including those in the beta lactam class. It was also observed in our study that the strains of *Salmonella* spp. isolated from the pig effluents had better susceptibility to gentamicin (85%), an aminoglycoside and a clinically-relevant protein synthesis inhibitor used for treating bacterial related infections. Gentamicin is a typical aminoglycoside that can be clinically combined with vancomycin and penicillin (both cell wall synthesis inhibitors) to treat bacterial related infections including those caused by anaerobic organisms. In contrast to our study, cephalosporin resistant *Salmonella* was isolated in Spain from fecal matter of 7-day-old piglets that were on conventional farms without any previous use of ceftiofur, a third-generation cephalosporin licensed for use in veterinary medicine (Cameron-Veas *et al*., 2018); thus signifying a possible horizontal gene transmission of MDR mobile genetic elements (AMR genes) responsible for the resistance of the organism to ceftiofur. In a previous related study conducted in the United Kingdom, antibiotic-resistant *Salmonella* isolated from swine wastes and farm surface waters was found to be highly susceptible to of resistance. cephalosporin (Casanova *et al*., 2019). Furthermore, the isolates of *Salmonella* spp. investigated in this study were found to be multiply resistant, and showed high levels of resistance to at least three (3) antibiotics of different classes. This further highlights the alarming levels of resistance reported in members of the *Enterobacteriaceae* family of which *Salmonella* spp. is a notable member of the enteric bacteria known globally to be implicated in foodborne and other bacterial infections (Koch *et al*., 2017; Koch *et al*., 2017; Shaban *et al*., 2014). The results of the multiple antibiotic resistance profiles of the strains of *Salmonella* spp. investigated in this study is comparable to what has been previously reported in China (Jiang *et al*., 2019), Spain (Cameron-Veas *et al*., 2018), Czech Republic (Sisak et al., 2006), Canada (Rosengren *et al*., 2008), Denmark (Baptista *et al*., 2009) and the United States of America (Duffy *et al*., 2005) – where increasing levels of multidrug resistance have been confirmed in pigs and other pig-associated materials and equipment. It is well established that the widespread application of antibiotics to animal populations raised for food, including chickens, cattle, and pigs, selects for resistance and contributes to the evolution of those pathogens (Koch *et al*., 2017). These antibiotic resistant pathogens could be transmitted to humans zoonotically, either through consumption, contacts with animal effluents or through body contacts with the animals during care, feeding or slaughter. Our preliminary results have shown that the *Salmonella* spp. investigated in this study were found to be multidrug resistant to commonly used first- and second-line antibiotics; nonetheless some last-line antibiotics including those in the cephalosporin family were found to be very effective in inhibiting the growth of the *Salmonella* spp. The reduced susceptibility of *Salmonella* spp. to some first- and second-line antibiotics as reported in this study portends a grave danger to public health and calls for further investigation to determine the actual disease burden of AMR caused by MDR bacteria including antibiotic-resistant *Salmonella* spp. in the propagation of pigs and other animals in Nigeria.

CONCLUSION

The results of this study have shown that antibiotic resistance, particularly in the livestock sector is a growing problem that requires further investigation in Nigeria. We have presumptively shown in this study that *Salmonella* spp. from pigs effluents are multiply resistant to some commonly used antibiotics in clinical medicine. Strains of *Salmonella* spp. that are highly resistant could further complicate empirical antibiotic therapy in scenarios where these pathogens are implicated in a public health disease outbreak. Studies like ours can further guide the Nigerian government about putting in place regulatory and practice policy changes that will help to protect both animal and human lives while ensuring antimicrobial stewardship and reduced antibiotic usage in livestock production in the country. We recommend a holistic review (of the extent) of antibiotic usage in livestock production in Nigeria, particularly in pig farms with the overarching goal(s) being to develop alternative approaches to the use of antibiotics in pig farms – which is known to promote the evolution and transmission of antibiotic resistant bacteria such as *Salmonella* spp. in both human and animal populations.

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