ACCESS MICROBIOLOGY

SHORT COMMUNICATION

Mamfe *et al.*, *Access Microbiology* 2021;3:000293

DOI 10.1099/acmi.0.000293

MICROBIOLOGY SOCIETY

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Phenotypic detection, antimicrobial susceptibility and virulence profile of staphylococci in the pig production setting, Makurdi, Nigeria

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Abstract

Livestock, particularly pigs, have increasingly been recognized as important reservoirs for zoonotic transmission of pathogenic bacteria, including staphylococci. Livestock production systems in developing countries of sub-Saharan Africa, including Nigeria, are characterized by high misuse/abuse of antimicrobials and a close association between humans and these animals, which promotes the emergence and transmission of resistant and potentially virulent bacteria. In the present study, we investigated the occurrence and characteristics (species distribution, virulence and resistance profile) of staphylococci from smallholder backyard pig farms, slaughter slabs and pig handlers in Makurdi, Nigeria. A total of 330 nasal swabs originating from 300 pigs and 30 in-contact humans were collected and processed. One hundred and thirteen samples [34.2%; 95% confidence interval (CI): 29.1–39.6] comprising 103 (34.3%; 95% CI: 29.0–40.0) and 10 (33.3%; 95% CI: 17.3–52.8%) samples from pigs and humans, respectively, were positive for staphylococci, yielding 120 isolates (pigs *n*=110, humans *n*=10). The 120 isolates were distributed into 15 species with *Staphylococcus aureus* (*n*=25) followed by *Staphylococcus cohnii* (*n*=19) and *Staphylococcus sciuri* (*n*=14) occurring more frequently. All isolates were resistant to *β*-lactam (100%) antibiotics. Resistance to some critical antimicrobials, including linezolid (22%), vancomycin (19.2%), gentamicin (7.5%) and the fluoroquinolones ciprofloxacin (75.8%) and enrofloxacin (66.7%), was also observed. Majority (99.2%) of the isolates displayed a multidrug resistance phenotype with the AMP-C-CIP-E-ENR-FOX-OX-P-S-SXT-TE phenotype being predominant. Overall, 70% of the isolates expressed the methicillin resistance phenotype, out of which 20% (*n*=17) were MRSA. Resistance to serum bactericidal activity and biofilm production were respectively observed in 45 (100%) and 5 (11.3%) of the coagulase-positive staphylococci. Our findings demonstrated the occurrence of a high diversity of staphylococci expressing multidrug resistance and potentially virulent phenotypes among healthy swine and pig handlers in small-scale backyard farms in North-Central Nigeria. These findings underscore the potential role of pig production settings in the emergence and dissemination of potentially virulent staphylococci and the importance of the development of antimicrobial resistance monitoring systems/implementation of control measures in developing countries. Proper hygienic practices and control of indiscriminate use and misuse of antibiotics are recommended.

INTRODUCTION

Livestock production settings represent an important hotspot for the emergence of virulent and resistant bacteria and may serve as a pool for their spread to other animals, farm workers and inhabitants of the immediate/surrounding community, and contamination of the environment [1]. The risk of acquiring these pathogens is further increased in developing countries, including Nigeria, where animals are increasingly being raised within or in close proximity to human dwellings, biosecurity practices are poor or nonexistent, and wastes from farms are discharged without treatment into the surrounding community [2, 3].

Staphylococci, although commonly regarded as a commensal of the mucosal surfaces of healthy humans and animals, are

Received 01 September 2021; Accepted 24 October 2021; Published 09 December 2021

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Keywords: antimicrobial resistance; humans; MRSA; Nigeria; pigs; staphylococci.

Abbreviations: AMP, ampicillin; C, chloramphenicol; CIP, ciprofloxacin; CoNS, coagulase negative staphylococci; CoPS, coagulase positive staphylococci; E, erythromycin; ENR, enrofloxacin; FOX, cefoxitin; LZD, linezolid; MRS, methicillin resistant staphylococci; MRSA, methicillin resistant *Staphylococcus aureus*; OX, oxacillin; P, penicillin; S, streptomycin; SXT, sulphamethaxazole-trimethoprim; TE, tetracycline.

Five supplementary tables are available with the online version of this article.

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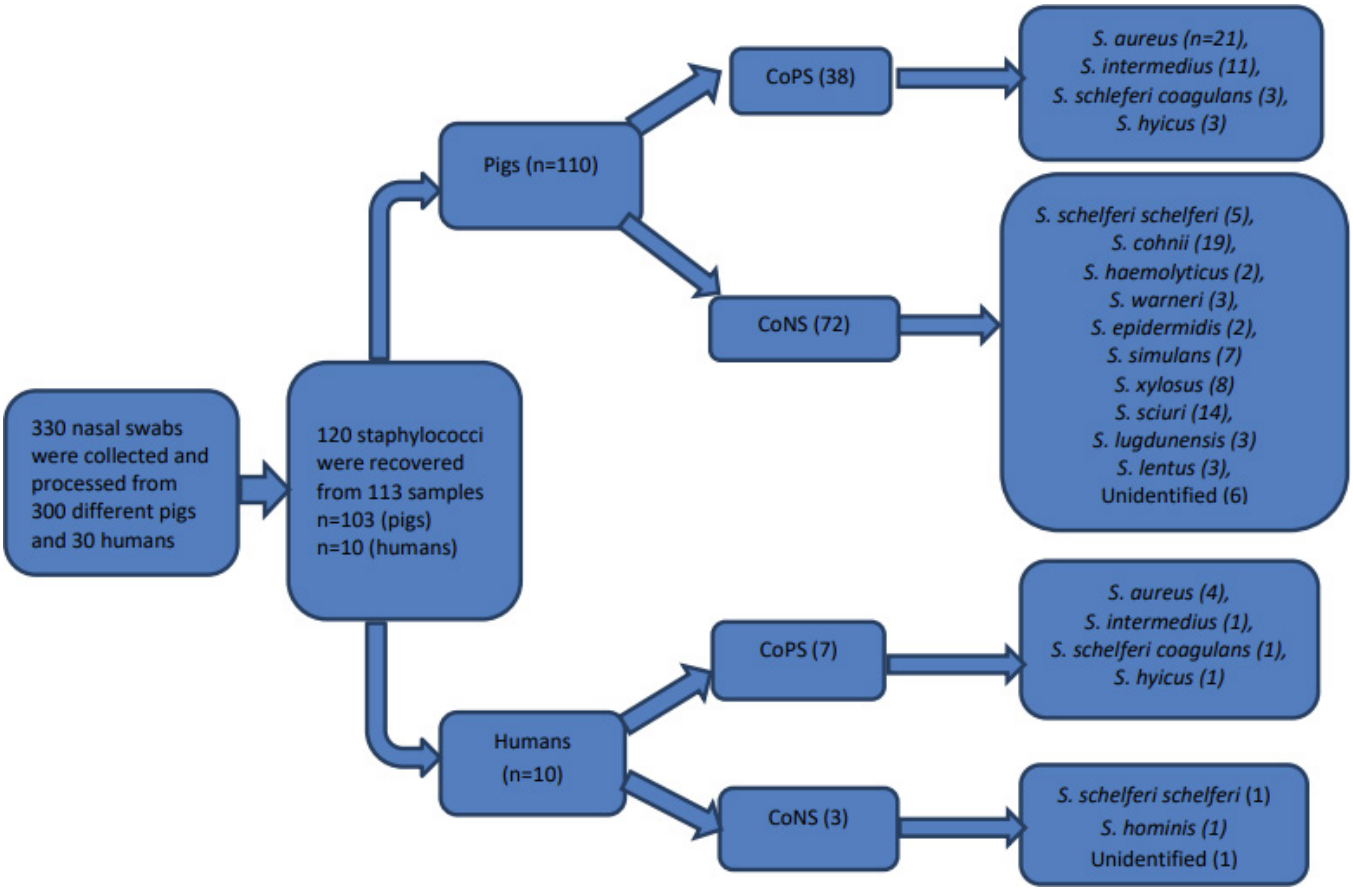

Fig. 1. Prevalence and species distribution of staphylococci from nasal swabs of pig and human handlers in the pig production setting, Makurdi, Nigeria.

also important opportunistic pathogens implicated in a vast array of infections [4]. Many studies have revealed the occurrence and exchange of indistinguishable staphylococci clones, particularly methicillin-resistant *Staphylococcus aureus*, between animals and humans, suggesting zoonoses. There is, however, limited information – particularly in Nigeria – on the staphylococcal populations inhabiting the nares of pigs, how they relate to those in their handlers and the pathogenic potential of these isolates. Current knowledge on staphylococci from pigs and humans in Nigeria is largely focused on *S. aureus* and its methicillin-resistant strains (MRSA) [5–7], neglecting the role of other staphylococcal species. Although *S. aureus* is the most important pathogen among all staphylococcal species, other staphylococci have in recent times been increasingly recognized and associated with several human and animal diseases and the spread of resistant determinants, including the methicillin resistance genes [8, 9]. More significantly, methicillin-resistant staphylococci (MRS) other than *S. aureus* have been reported in food animals and animal products [10].

The North-Central States, including Benue, are among the hubs for pig production, with pigs playing an essential role in the food security and socio-cultural lives of the inhabitants of this area. Pork is among the most frequently consumed meat products in the study area and pigs are predominantly raised in backyard farms (within or in close proximity to human dwellings) rather than commercial farms. Similar to other resource-limited countries in sub-Saharan Africa, antimicrobial use and abuse is widespread in the pig production settings in this region, and the farming system characterized by poor or non-existent biosecurity practices and no established antimicrobial surveillance system. The characteristics of this predominant pig production system and its practices promote the emergence of resistant bacteria and facilitate potential transmission between humans and animals as well as contamination of the environment. However, there is a knowledge gap concerning how this production system may influence the emergence and dissemination of antimicrobial resistance (AMR) in zoonotic foodborne bacteria in the country. We hypothesize a high circulation rate for potentially virulent and antimicrobial-resistant staphylococci among pigs in this area and that the resistogram will be a reflection of the common antimicrobials used in livestock production in the study area. The purpose of the present study was to investigate staphylococcal carriage and species diversity among apparently healthy pigs and their human handlers and to further

Table 1. Antimicrobial resistance profiles of staphylococci isolated from pigs and their handlers in Makurdi, Benue State, Nigeria

Antimicrobial		No of resistant isolates (%)		
Category	**Agent**	**Pigs (*n*=110)**	**Humans (*n*=10)**	**Total (*n*=120)**
Aminoglycosides	Gentamicin	8 (7.3)	1 (10)	9 (7.5)
	Streptomycin	65 (59.1)	8 (80)	73 (60.8)
Cephalosporin	Cefoxitin	82 (74.5)	9 (90)	91 (75.8)
Fluoroquinolones	Ciprofloxacin	84 (76.4)	7 (70)	91 (75.8)
	Enrofloxacin	71 (64.5)	9 (90)	80 (66.7)
Glycopeptides	Vancomycin	22 (20)	1 (10)	23 (19.2)
Macrolides	Erythromycin	97 (81.2)	10 (100)	107 (82.2)
Oxazolidone	Linezolid	22 (20)	4 (40)	26 (21.6)
Penicillins	Ampicillin	110 (100)	10 (100)	120 (100)
	Penicillin	89 (80.9)	9 (90)	98 (81.7)
	Oxacillin	110 (100)	10 (100)	120 (100)
Phenicols	Chloramphenicol	73 (66.4)	9 (90)	82 (68.3)
Folate	Supha-methoxazole	60 (54.4)	9 (90)	69 (57.5)
Tetracycline	Tetracycline	89 (80.9)	9 (90)	98 (81.7)

investigate the antimicrobial resistance and virulence profile of the isolates phenotypically.

METHODS

Study site and design

A cross-sectional survey was conducted between April and December 2019 across pig farms/holdings and pig slaughter slabs in Makurdi, North-Central, Nigeria, one of the major pig-producing areas in the country. Participating farms and individuals were selected based on convenience, i.e. willingness of the farm owner to allow his farm to be included and handlers/workers to participate in the study.

Ethical approval

The study was approved by the Research and Ethics Committee of Benue State Ministry of Health and Human Services (MOH/STA/204/VOL.1/128). Informed consent was also obtained from all human participants before their inclusion in the study.

Sampling and sample size

The minimum number of pigs to be sampled (sample size) was estimated to be 78 using a 95% confidence level, an expected prevalence of 5.3% [11] and desired precision of 5%. Nasal swabs were collected from at most 30 different pigs in farms with more than 30 pigs, while in farms with small herds, i.e. less than 30 pigs, all the pigs were sampled. For humans, at least one individual working with the pigs was sampled. A total of 330 nasal swabs were collected randomly from 300 pigs and 30 humans in 19 pig farms and 3 slaughter slabs. Nasal swabs were collected using moistened sterile swabs sticks, which were inserted into the nares and rolled. All the nasal swabs collected were inoculated directly into an enrichment broth [tryptone soya broth (Oxoid, UK) supplemented with 6.5% NaCl] and placed in a cold box for onwards transport to the laboratory within 2–4 h for processing.

Isolation and identification of staphylococci

The nasal swabs were processed for isolation and identification of staphylococci using standard bacteriological techniques. Briefly, on arrival in the laboratory all the samples (enrichment broth inoculated with the swabs on the field) were incubated at 37 °C for 18–24 h followed by inoculation of a loopful of the enrichment broth onto mannitol salt agar (MSA) (Oxoid, UK) for selective isolation of staphylococci. The MSA plates were inoculated for 18–24 h and colonies with staphylococcal morphology (pink or yellow) were picked and sub-cultured to purity on nutrient agar.

Presumptive staphylococcal colonies were identified using Gram staining and the catalase test and speciated using a combination of recommended biochemical tests [12], including DNase tests, slide (clumping factor) and tube coagulase tests, sensitivity to novobiocin (5 µg), ornithine decarboxylation, acetoin production, urease activity, and aerobic fermentation of mannitol, mannose, sucrose, xylose, trehalose and lactose. Previously published simplified flow charts for speciation of staphylococci based on the result of these listed tests were also used to ease the speciation for coagulase-positive and -negative isolates [12, 13] and the chart by Goyal *et al.* [14] for the coagulase-negative isolates

Table 2. Distribution of methicillin-resistant staphylococci (MRS) isolated from pigs and their handlers in Makurdi, Benue State, Nigeria

Staphylococcus species	No. of methicillin-resistant		
	Pigs	Humans	Total
S. aureus	13	4	17
S. intermedius	7	1	8
S. schelferi coagulans	1	0	1
S. hyicus	1	1	1
S. schelferi schelferi	3	1	4
S. haemolyticus	2	–	2
S. warneri	3	–	3
S. cohnii	17	–	17
S. epidermidis	2	–	2
S. simulans	5	–	5
S. xylosus	7	–	7
S. sciuri	12	–	12
S. lungdensis	1	–	1
S. lentus	3	–	3
S. hominis	–	1	1

only. The *S. aureus* species were further confirmed using the Staphytect plus latex agglutination kit (Oxoid, UK).

Antimicrobial susceptibility testing

Susceptibility of the isolates to antibiotics was assessed by the Kirby–Bauer disc diffusion method according to the Clinical and Laboratory standards Institute (CLSI) guidelines [15, 16] using Mueller–Hinton agar and commercial antimicrobial discs (Oxoid, UK). The antimicrobial agents used include: ampicillin (10 µg), cefoxitin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), erythromycin (15 µg), linezolid (30 µg), oxacillin (1 µg), penicillin (10 µg), streptomycin (30 µg), sulphamethaxozole–trimethroprim (25 µg), tetracycline (30 µg) and gentamicin (30 µg). Vancomycin susceptibility was determined by the minimum inhibitory concentration using the agar dilution method. The susceptibility result was determined by measurement of the zone of inhibition and the result was interpreted as susceptible, intermediate or resistant for each antibiotic according to the CLSI guidelines for veterinary isolates [15] and human isolates [16]. Intermediate isolates were classified as resistant. Cefoxitin- or oxacillin-resistant isolates were categorized as methicillin-resistant staphylococci (MRS in general) depending on the species [15, 16]. Isolates resistant to ≥3 different classes of antimicrobials were classified as multidrug-resistant (MDR) [17].

Virulence profile

The coagulase-positive staphylococcal isolates were tested for expression of biofilm and resistance to serum bactericidal activity. Biofilm production was assayed using the Congo red dye method as previously described by Kaiser *et al.* [18], while resistance to serum bactericidal activity was assayed using the method described by King *et al.* [19].

RESULTS

Occurrence and species distribution of staphylococci

Of the 330 samples collected from pigs (*n*=300) and humans (*n*=30), staphylococci were isolated from 113 samples comprising 103 (34.3%: CI: 31.2–42.4%) from pigs and 10 (33.3% 95% CI: 17.3–52.8%) from humans. A total of 120 staphylococci were recovered from the 113 positive samples. Coagulase-negative staphylococci (CoNS) were more predominant, particularly among the pig (*n*=72) isolates, compared to coagulase-positive (CoPS) species (*n*=38). The 120 staphylococci isolated were distributed into 15 species while the species identity of 7 coagulase-negative isolates could not be completely verified, i.e. could not be assigned species (Fig. 1). *S. aureus* was the most common species in both pigs (*n*=21; 21/110) and humans (*n*=4; 4/10), followed by the coagulase-negative *Staphylococcus cohnii* (*n*=19; 19/110) and *Staphylococcus sciuri* (*n*=14; 14/110) in pigs, respectively (Fig. 1).

Antimicrobial susceptibility

The antimicrobial susceptibility result is presented in Table 1. All the isolates (100%) tested were resistant to oxacillin and ampicillin. Varying resistance rates ranging from as low as 7.5% for gentamicin to 82.2% for erythromycin, were recorded for the other antimicrobials. Resistance to the high priority critical antimicrobials, including vancomycin (19.2%) and linezolid (21.6%), were also recorded (Table 1). The result for each species is presented in the Tables S1–S3 (available in the online version of this article).

Multidrug resistance

Majority (99.2%) of the isolates were multidrug-resistant (resistant to ≥3 different classes of antimicrobials), with the most resistant being to all 11 antibiotic classes. One *S. sciuri* isolate from pigs was resistant to all 15 antimicrobial agents tested. AMP-CIP-E-ENR-FOX-OX-P-S-TE was the most predominant (*n*=6) phenotype among the pig isolates compared to AMP-C-CIP-E-ENR-FOX-LZD-OX-P-S-SXT-TE (*n*=2) and AMP-C-CIP-E-ENR-FOX-OX-P-S-SXT-TE (*n*=2) in the human isolates (Tables S4a–c and S5).

All the isolates had a multiple antibiotic resistance (MAR) index ≥0.2. The values of the MAR index for the *Staphylococcus* isolates ranged from 0.2 to 1 with a mean value of 0.65. (Tables S4a–c and S5).

Table 3. Frequency of expression of the virulence trait among coagulase-positive staphylococci isolated from pig production settings in Makurdi, Benue State, Nigeria

Staphylococcus species	Serum bactericidal resistance			Biofilm production		
	Pigs	Humans	Total	Pigs	Humans	Total
S. aureus	21	4	25	3	0	3
S. intermedius	11	1	12	2	0	2
S. schleiferi coagulans	3	1	4	0	0	0
S. hyicus	3	1	4	0	0	0

Methicillin resistance

Of the 120 isolates, 84 (70%) distributed across the 15 species were methicillin-resistant staphylococci (MRS) (Table 2). Although both coagulase-positive and -negative staphylococci expressed the methicillin resistance phenotype, overall coagulase-negative staphylococci species expressed the phenotype more frequently than coagulase-positive species, particularly in pigs. The coagulase-negative *S. cohnii* (*n*=17) followed by the coagulase-positive *S. aureus* (MRSA) were the predominant methicillin-resistant species in pigs, while *S. aureus* (*n*=4) was the predominant one in humans.

Virulence profile

The frequency of expression of virulence traits by the coagulase-positive staphylococci species is presented in Table 3. All (45/45) of the coagulase-positive staphylococci were resistant to the bactericidal action of serum, while only 11.1% (5/45) produced biofilm.

DISCUSSION

The emergence and spread of multidrug-resistant bacteria, particularly in livestock production settings, is increasingly a source of public health concern globally. Swine husbandry has been recognized as an important reservoir for the emergence and dissemination of resistant and virulent *Staphylococcus* species. In the present study, we observed the occurrence of diverse species of staphylococci, the majority of which were multidrug-resistant and possess the potential to cause clinical infections. These findings suggest that the pig production setting in this area could serve as an important source for the dissemination of antimicrobial-resistant and potentially virulent staphylococci. Similar species distributions have previously been reported in pigs, pork and in-contact humans in sub-Saharan Africa [10, 20–22].

All the isolates phenotypically multidrug-resistant (MDR), had an MAR index of ≥0.2, which suggests that the isolates originated from a source where antibiotics are used frequently [23]. The high rates of resistance observed towards the β-lactams, penicillins, tetracyclines and macrolides are consistent with the findings from previous studies in animals and animal products, including pigs and pork [10, 21, 22, 24], poultry [25], cattle [26], camels [27] and humans [11, 21, 25] in Nigeria. This high rate of resistance may not be coincidental, as we hypothesize that it is linked to the well-documented widespread, heavy and unregulated use of these agents in pig production across the country for prophylaxis and therapy [28]. In agreement with a previous report by Ogunleye and Okunlade [29], a high rate (68%) of resistance to chloramphenicol, a drug whose use in veterinary medicine has been banned worldwide, including in Nigeria, was observed. This observation may also be attributed to the widespread use of the drug in animal production in Nigeria in spite of the ban [28, 30–32]. This further supports the need for the roll out of an appropriate programme and enforcement of regulations on drug use and access in the country. Selection pressure from the abusive use of antimicrobials has been shown to facilitate the emergence of resistant strains [32]. The multidrug resistance phenotype expressed by the isolates is particularly worrying, as it may limit the available therapeutic armamentarium in the event of infection by these pathogens.

Although the categorization of *Staphylococcus aureus* as methicillin-resistant (MRSA) in the present study was purely based on the phenotypic characterization, the findings agree with other studies that reported MRSA from pigs and pig handlers in Nigeria [5–7]

Surprisingly, some of the isolates were resistant to two 'last resort' antibiotics, vancomycin and linezolid, which are not commonly used in livestock production or human medicine in Nigeria. There is a recent increase in the frequency of detection of linezolid- and vancomycin-resistant Gram-positive bacteria in livestock in sub-Saharan Africa. The increase in linezolid-resistant isolates may likely be a result of the recent increase in use of florfernicol in many farms across Nigeria [33]. Florfernicol has been shown to provide selective pressure for the emergence of linezolid-resistant strains [34]. Some of the genes mediating resistance to these last-resort drugs have been reported to be located/carried on mobile genetic elements, which may facilitate their rapid dissemination [35].

Notably, all the coagulase-positive species from both humans and pigs displayed resistance to serum bactericidal activity and a few produced biofilm. Biofilm formation is known to confer a fitness advantage on bacteria by enhancing their survivability and resistance to antibiotics and facilitating their ability to acquire virulence and antibiotic resistance genes during horizontal gene transmission due to their high microbial density [36].

Collectively, the MDR, resistance to serum bactericidal activity and biofilm production ability of the isolates could make them difficult to control in swine farms and, once introduced into the community and hospital environments, increase their capacity to survive and proliferate in these environments. Additionally, species with these properties could be disseminated into the environment via manure and other wastes originating from the farms and slaughter facilities.

The detection of resistant and potentially virulent staphylococci in pigs and exposed workers in the farms and slaughter facilities is of great public health significance and highlights a serious food safety threat, as these facilities, particularly the slaughter slabs, serve as the major source of pork for the community. These findings therefore further suggest that pig farms, slaughter facilities and workers in the study location may constitute a potential reservoir and source for spread of foodborne staphylococcal infections, highlighting the importance of the implementation of food safety measures and regulations concerning antimicrobial stewardship in livestock production settings in the study area.

This study, however, has some limitations, particularly the inclusion criteria for sampling, with the willingness of the farm owner or worker to participate being required, which may have introduced some bias. Further, our inability to carry out molecular characterization of the isolates limited our inferences.

CONCLUSION

This study demonstrates that the pig production settings in the study area may constitute a reservoir for the emergence and dissemination of virulent and antibiotic-resistant staphylococci. Further research using high-resolution genomics is required to understand the molecular epidemiology, likelihood of exchange or spillover from the pig population to humans. The findings from our study also suggest the need for increased antimicrobial surveillance in the swine production environment in the area to mitigate the increased emergence and dissemination of resistant strains.

Funding information
This work received no specific grant from any funding agency.

Author contributions
Conceptualization: L.M.M., C.A.A., E.O.N. Methodology: L.M.M., C.A.A., E.O.N. Validation: L.M.M., C.A.A., E.O.N. Formal analysis: L.M.M. Investigation: L.M.M. Resources: C.A.A., E.O.N. Data curation: L.M.M. Writing – original draft: L.M.M. and E.O.N. Writing – review and editing: L.M.M., C.A.A., E.O.N. Supervision: C.A.A. and E.O.N.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The study was approved by the Research and Ethics Committee of Benue State Ministry of Health and Human Services (MOH/STA/204 /VOL.1/128). Informed consent was also obtained from all human participants before their inclusion in the study.

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