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Protected but not from Contamination: Antimicrobial Resistance Profiles of Bacteria from Birds in a Ghanaian Forest Protected Area

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ABSTRACT: Resistance to antimicrobial agents is a growing concern in public health. It has been reported in wildlife from several places in the world though wild animals are not normally exposed to clinically used antimicrobial agents. Despite this, very little research has been done in Ghana to determine antimicrobial resistance in wild animals, particularly those in protected areas. In this study, the presence of colistin resistant and multidrug resistant (MDR) gram-negative bacteria in cloacal swabs of wild birds captured in a Ghanaian forest protected area were evaluated. A total of 195 isolates from 138 individual birds were obtained, identified and tested for resistance to colistin. The colistin-resistant isolates were subsequently tested for multidrug resistance to 4 other antimicrobial agents (Oxytetracycline, Streptomycin, Ampicillin and Ciprofloxacin). Colistin resistance was observed in 6.5% (9/138) of the birds and this was seen in only birds that were sampled close to the reception area of the protected area. About 50% of the colistin-resistant isolates were multidrug resistant. AMR isolates were obtained from birds that have been documented to show an insectivorous or omnivorous feeding preference. Data obtained from the study suggests that AMR and MDR occurred in wild birds from the Conservation Area and supports the claim that proximity to human impacted habitats (settlements/farmlands) increased the likelihood of carriage of AMR. Though the routes of transmission remain unclear, there is potential for spread from the wild birds to other wild/domestic animals and possibly back to humans.

KEYWORDS: Antibiotic resistance, wild birds, disc diffusion, colistin, forest

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Introduction

Protected areas (PAs) are legally acquired areas dedicated and managed for the long term conservation of nature.[1] They are the main focal areas for the conservation and management of biodiversity.[2] Also, they serve as refugia to wild animals in areas where they are exploited for meat, particularly in Central and West Africa.[3] As a result, PAs are considered to be sites where wild animals experience minimal human disturbance. This claim is supported by findings from studies that have shown that bacteria isolated from wildlife in remote areas show little or no evidence of antibiotic resistance.[4–7] On the other hand, wild animals sampled from areas with relatively high anthropogenic influence have shown high resistance to antimicrobial agents.[8–10] It may be inferred from the above that the wild animals in protected areas also have minimal or no antibiotic resistance.

Antimicrobial resistance is a serious concern with major implications for human and animal health.[11] Once wild animals acquire resistant bacteria, they may become bioindicators or reservoirs of resistant bacteria.[12] They can also reintroduce resistant bacteria to humans and domestic animals via contamination of shared soil and water sources.[13] The prevalence of antibiotic-resistant bacteria in wild animals appears to be dependent on a variety of factors including foraging strategies and the type of habitat where the animal was sampled. However, contact with a human-influenced habitat remains a major factor leading to the acquisition of resistant bacteria.[14]

The relatively high frequency of possible contact that wild birds have with humans and other animals due to the nature of their mobility makes them ideal animals for investigating the exchange of microbes or pathogens in the human-wildlife interface.

Very little information is available on the prevalence of antibiotic-resistant bacteria in wild animals inhabiting protected areas. Though threats of transmission to/from wild animals have been observed in studies from several countries,[5,9,15-17] there is little information on the occurrence of antimicrobial resistance in wild animals from Ghana. For example, a study inferred antibiotic resistant bacteria transfer to wild birds that use landfill sites by examining soil collected from the land fill sites.[18] Against the backdrop that there is little information

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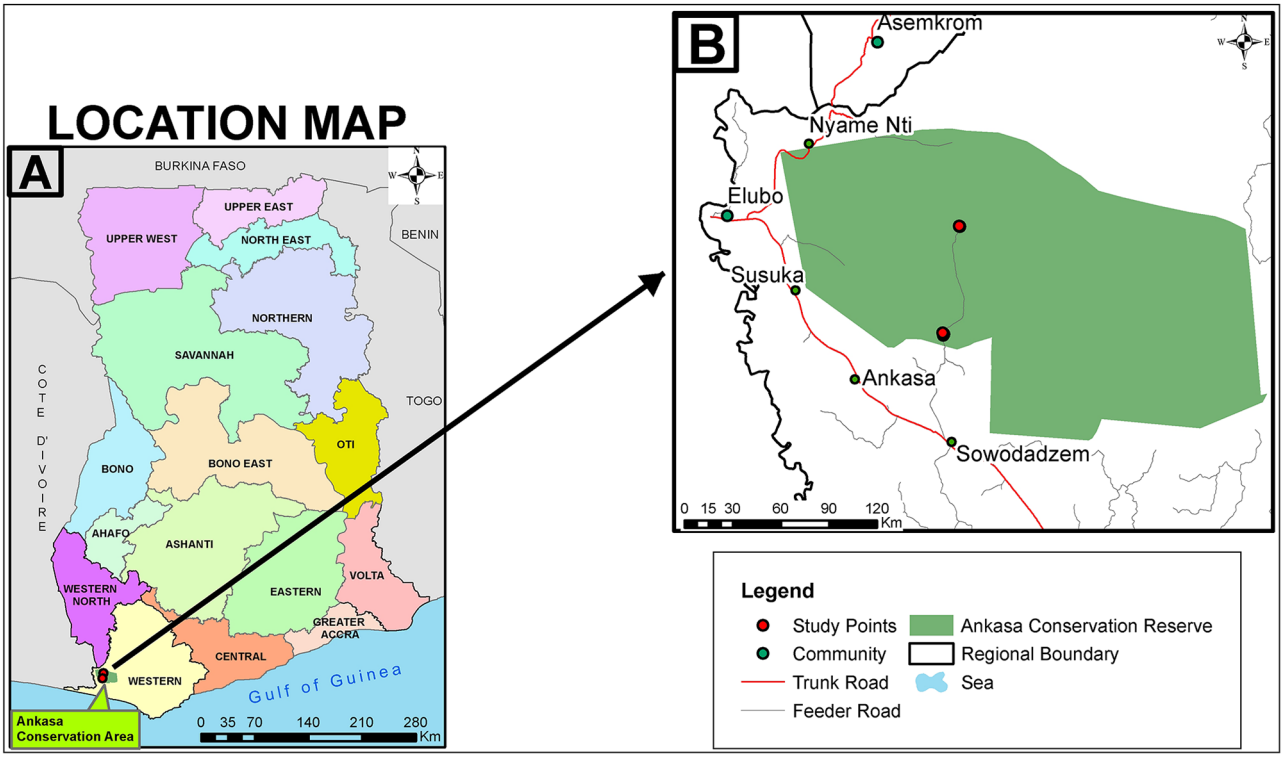


Figure 1. A map of Ghana showing the Ankasa Conservation Area were the study was carried out. (A) administrative map of Ghana; (B) Ankasa Conservation Area showing survey sites.

available on antibiotic-resistant bacteria in wild birds from Ghana, this study examined the cloacal microbiota of wild birds from the Ankasa Conservation Area for presence of gram-negative bacteria, and determined their antimicrobial resistance profiles. Specifically, we determined antimicrobial sensitivity to colistin, one of the last resort antimicrobial agents used in the treatment of multidrug resistant infections caused by gram-negative bacteria. Subsequently, colistin-resistant isolates were evaluated for multidrug resistance.

Materials and Methods

Study site description

Birds were trapped from the Ankasa Conservation Area (ACA), a protected area comprising the Nini Suhien National Park and the Ankasa Resource Reserve. It is located in the Western Region of Ghana (Figure 1). The Conservation Area is a wet evergreen forest and covers an area of approximately 50,900 ha of land.[19] It is relatively pristine as the Nini Suhien part of the area remains untouched. The area is biodiverse and home to several species of plants and animals including 800 vascular plants, 600 butterfly species, 43 mammal species and 10 primate species.[20] Two roads and a powerline run through a larger portion of the Conservation Area. The old Nkwanta village and Nkwanta camp serve as corridors where non-forest bird species could be found. Settlements around the reception area belonged to inhabitants of the Nkwanta village (formally located within the Conservation Area) and workers of the Forestry Commission, Ghana. The inhabitants of the Nkwanta village were engaged mainly in farming; their farms were located at the periphery of the reception area. They produced cash crops (coconut, cocoa and palm plantations), edible crops (banana and pineapple) and food crops (plantain, cassava, vegetables, yam).[21]

Trapping of birds

Permission to collect samples in the study area was obtained from the Wildlife Division of the Ghana Forestry Commission. Ethical considerations regarding the study were approved by the Ethics Committee of the college of Basic and Applied Science, University of Ghana (ECBAS 003/17-18). Birds were captured using nylon mist-nets between December 2017 and May 2018 in two locations within the protected area: around the Nkwanta camp and about 300 to 800 m away from the reception area (Figure 1). Captured birds were removed from the nets and each placed in a cotton bird bag. The bags were sent to a processing area immediately for sample collection. The processing area was about 5 minutes away by foot from where the birds were captured. Each bird was identified, assessed for health by physical examination for any signs of disease. Sex (for sexually dimorphic species) and age (adult or juvenile) of each bird were recorded. The birds were not anesthetized or sedated during sampling as this was a very harmless procedure. All captured birds were ringed using the Ghana ringing scheme[22] to help identify recaptures. In order not to alter the way the birds interact with their natural environment, they were not housed and were released unharmed to the locations they were captured within 30 minutes from time of capture.

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Sample collection

Sterile swabs were used in collecting cloacal swabs from trapped birds. Non-duplicate cloacal swab sample was collected from each bird. The swabs were inoculated into 200 µl phosphate buffered saline (PBS); the samples were kept at −20°C for the 2 days spent on each trip and transported on ice to the Biotechnology laboratory, University of Ghana for further processing.

Isolation of bacteria

Each swab was streaked on MacConkey agar, and the agar plates were incubated at 35°C for 18 to 20 hours aerobically. The agar plates were inspected for colonies and distinct morphotypes were picked per sample and purified individually. The pure colonies of bacteria were inoculated onto soft nutrient agar in microtubes and stored at room temperature for further examinations. Isolates were identified by morphological characteristics, gram staining, motility test and biochemical tests (indole, catalase, oxidase, urease, Triple Sugar Iron (TSI), Methyl-Red-Voges-Proskauer and citrate).[23]

DNA extraction and sequencing of bacteria isolates

The genera of 24 resistant isolates were confirmed by sequencing of the 16S rRNA. First, whole DNA was extracted from the isolates using the boiling method.[24] To identify the isolates, PCR amplification (size of amplicon was 919 bp), purification and sequencing of the 16S rRNA were performed.[25,26] Each nucleotide was assembled with SNAPGENE (GSL Biotech, available at snapgene.com) and BioEdit (Version 7.2.5, available at bioedit.software.informer.com/7.2/) softwares. A partial sequence was used to compare 16S rRNA gene sequences in the nucleotide Basic Local Alignment Sequence Tools (BLAST) geneBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for similarities. Sequences with similarity percentage ⩾99% were considered closely related species in terms of genus.

Antimicrobial susceptibility test

Antimicrobial susceptibility for 195 isolates was determined by supplementing molten MacConkey agar with colistin sulfate (Fujifilm Wako Pure Chemical Corporation, Japan) (2 µg/ml).[27] The solidified medium was streaked with a bacteria isolate and incubated for 24 hours at 37°C. Agar plate supplemented with colistin that showed bacteria growth were presumed to be resistant to colistin. The presumptive colistin-resistant isolates were subsequently subjected to multidrug resistance test using the disc diffusion method.

The Kirby Bauer disc diffusion method was used for the detection of multidrug resistant isolates following guidelines of the Clinical and Laboratory Standards Institute.[28] A total of 4 commercially prepared antimicrobial disc (6 mm) were used for the disc diffusion test: ampicillin (AMP 10 µg), oxytetracycline (OTC 30 µg), streptomycin (SM 10 µg) and ciprofloxacin (CIP 5 µg) (Nippon Becton Dickinson Co. Ltd., Fukushima Prefecture, Japan). Three of the antimicrobial agents were selected because of their frequency as active agents in the composition of antibiotics given to farm animals in Ghana[29–32] and ciprofloxacin was selected because of its common use in the treatment of urinary tract infection[33,34] and cholera in Ghana.[31] The reference strain, *E. coli* ATCC 25 922, was used as control and the interpretation of the diameters of the zones of inhibition was determined using the CLSI recommendations (resistant, intermediate or susceptible).[35] A 150 mm Mueller-Hinton medium plate was swabbed with TSB, inoculated with each bacterial isolate and incubated to a turbidity of 0.5 McFarland standard.[36] The 4 antibiotic discs were placed on the inoculated plates and incubated for 35°C for 18 to 20 hours. The zones of inhibition around the antimicrobial disc including the 6 mm antimicrobial agent diameter were measured with Vernier calipers.

Data analysis

Bird species were categorized on the basis of where they were trapped in the forest (ie, 800 m from the reception area and 8 km away from the reception area).[21] To avoid the potential bias of using uneven numbers of isolates per sample, statistical comparisons were conducted at the faecal sample level where a sample was recorded as resistant when at least 1 of the isolates obtained from the faecal sample was resistant to an antimicrobial agent, and multidrug resistant when at least 1 of its isolates was resistant to 3 or more classes of antimicrobials. The data were analyzed using the Fishers exact test and Binomial test in R version 3.4.2.[37] Confidence intervals of resistance rates were also calculated. P-value $< .05$ statistical significance was set.

Results

Birds captured and isolation of gram-negative bacteria

Overall, 138 cloacal swabs were obtained from the forest birds (one swab from each bird). The birds belonged to 9 families and 20 species (Supplementary file). A total of 195 gram-negative bacteria isolates belonging to 10 genera were isolated, namely: *Shigella spp., Yersinia spp., Salmonella spp., Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Pseudomonas* spp., *Serratia* spp. and *Escherichia* spp. The numbers of isolates belonging to each bacteria genus and their prevalence are presented in Table 1.

Resistance to colistin

Overall 6.5% (n = 9, 95% CI 3.0-12.0%) of the birds (9/138) harboured colistin-resistant bacteria. Resistant isolates belonged to the genera *Citrobacter, Enterobacter, Escherichia* and *Klebsiella* (Table 2). A comparison of the prevalence of the 4 different bacteria genera (*Citrobacter, Enterobacter, Escherichia, Klebsiella*) in the microbiota of the birds showed no significant difference ($P = .406$).

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Table 1. Proportions of gram-negative enterobacteriaceae genera isolated from the forest birds.

BACTERIA GENERA	NUMBER OF ISOLATES	PROPORTION OF ALL ISOLATES (%)
Shigella spp.	8	4.10
Yersinia spp.	36	18.46
Salmonella spp.	8	4.10
Citrobacter spp.	16	8.21
Escherichia spp.	77	39.49
Enterobacter spp.	16	8.21
Klebsiella spp.	16	8.21
Proteus spp.	5	2.56
Pseudomonas spp.	8	4.10
Serratia spp.	5	2.56
Total	195	100

Colistin-resistant bacteria isolates were obtained from 6 bird species: Forest Robin, Icterine Greenbul, Olive Sunbird, Red-tailed Bristlebill, White-tailed Ant Thrush, Yellow-whiskered Greenbul (Table 2). In 2 of the bird species (Yellow-whiskered greenbul and Forest robin), a maximum of 3 colistin-resistant bacteria genera (*Enterobacter, Escherichia* and *Klebsiella*) were observed co-existing in an individual bird. All the colistin-resistant isolates were obtained from birds trapped around the reception area (300-800 m away).

Multidrug resistance

About 96% (23/24) of all the colistin-resistant isolates that were subjected to multidrug resistant tests showed resistance to at least one of the following antimicrobial agents: Ampicillin, Streptomycin, Oxytetracyline and Ciprofloxacin. Only one isolate (*Citrobacter* sp.) did not show resistance to any of the antimicrobial agents used for the multidrug resistance test. About Sixty-seven percent (16/24) showed resistance to 2 of the antimicrobial agents while 50% (12/24) were multidrug resistant.

About thirteen percent (3/24) of the isolates were resistant to Ciprofloxacin, 95.8% (23/24) to Ampicillin, 66.6% (16/24) to Oxytetracycline and 50% (12/24) to Streptomycin (Figure 2). There was a significant difference in resistant isolates to the 4 antimicrobial tested ($P < .05$).

About 68% (6/9) of the birds harboured multidrug resistant bacteria isolates. Multidrug resistance was recorded in 5 bird species: Yellow-whiskered Greenbul, Red-tailed Bristlebill, Olive Sunbird, Icterine Greenbul and Forest Robin.

Discussion

The findings of the study revealed that the forest birds captured harboured bacteria that belonged to 10 genera of gram-negative enterobacteriaceae, an indication that several species of bacteria may be present in the birds. Bacteria genera identified were similar to those isolated from wild birds associated with animal production and farmlands.[38]

Resistance to colistin was observed in 6.5% of the birds. It has also been reported in bacterial isolates from humans, food-producing animals, companion animals and the environment.[39,40] Being a last line antimicrobial agent for the treatment of pan-drug resistance caused by gram-negative bacteria, emergence of resistance to this antimicrobial is raising a lot of concerns worldwide.[41] The first case of colistin-resistance in wild birds was detected in a bacterial isolate from a migratory bird (European herring gull *Larus argentatus*) in 2016.[42] In this study, resistance to colistin was unanticipated, mainly because samples were collected from birds in a protected area where human-wildlife interaction is limited compared to migratory waterbirds such as gulls.

Antimicrobial resistance is a major concern, particularly in Africa where the climate is conducive to the survival of most bacteria in the environment, however, many countries on the continent lack regulations on the use of antimicrobial agents.[43] The non-therapeutic/sub-therapeutic usage of antimicrobial agents has been blamed for the spike in antimicrobial resistance.[44,45] In the case of Ghana, there was no policy on the use of antimicrobials until 2018.[42] Before the policy was launched, weak regulations existed for antimicrobial use for human but none for animals.[46] Some studies on the use of antimicrobial agents among poultry farmers in Ghana found active ingredients including colistin.[29,30] It is possible that some livestock farmers in the rural communities surrounding the protected area may be using these medicated feed to enhance their production, hence, the occurrence of colistin resistance only in isolates from birds that were sampled close to the reception area. In addition, farmers do not adhere strictly to withdrawal periods for antimicrobial usage in farm animals [29,30,47] making it highly possible for antibiotic residue transfer. Few studies have reported colistin resistance from clinical samples in Ghana,[48] suggesting the possibility that human population in surrounding communities of the protected area could be harbouring colistin-resistant bacteria from the consumption of meat from livestock with antimicrobial residue. These surrounding human populations could disseminate resistant bacteria via indiscriminate exposure of faecal matter as most of the communities had no places of convenience and often used a nearby bush.[21] Furthermore, resistance may be spread to other parts of the conservation area far from the reception area by wildlife rangers. These rangers go on routine patrols and spend days covering vast distances; they could shed resistant isolates via faecal matter/urine as there may not be places of convenience depending on where they find themselves in the protected area.

Some gram-negative bacterial species including, but not limited to, the following: *Burkhlderia cepacia* complex, *Edwardsiella tarta, Morganella morganii, Proteus* spp.,

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Table 2. Prevalence of colistin-resistant gram-negative enterobacteriaceae in each bird species.

BIRD SPECIES	BACTERIA GENERA ISOLATED	NO. OF COLISTIN-RESISTANT ISOLATES	PREVALENCE[A]	LIFE HISTORY STRATEGY[B]		
Forest Robin	*Escherichia* spp.	4	16.7 (1/6; 0.4-64.1)	FF/P	I	GU
Icterine Greenbul	*Citrobacter* spp.	3	33.3 (1/3; 0.8-90.6)	FF/P	I	LM
Olive Sunbird	*Escherichia* spp, *Citrobacter* spp.	4	10 (1/10; 0.3-44.5)	F/P	F-I-N	GU
Red-tailed Bristlebill	*Klebsiella* spp., *Citrobacter* spp.	4	33.3 (1/3; 0.8-90.6)	FF/P	O	GU
White-tailed Ant Thrush	*Enterobacter*spp.	1	33.3 (1/3; 0.8-90.6)	FF/P	I	GU
Yellow-whiskered Greenbul	*Enterobacter* spp, *Escherichia* spp, *Klebsiella* spp.	8	14.3 (4/28; 4.0-2.7)	F/P	O	GU
Total		24	6.5 (9/138; 3.0-2.0)			

F, frugivore; F/P, primary forest; FF/P, primary forest interior; GU, ground and understorey; I, insectivore; LM, lower and midstorey; N, nectarivore; O, omnivore.
[a](%) Prevalence of cloacal samples that harboured resistant bacteria (Number of birds that harboured resistant bacteria (n)/Total number of birds examined (N); exact binomial 95% confidence interval).
[b]Life history strategy obtained from literature.[32–37]

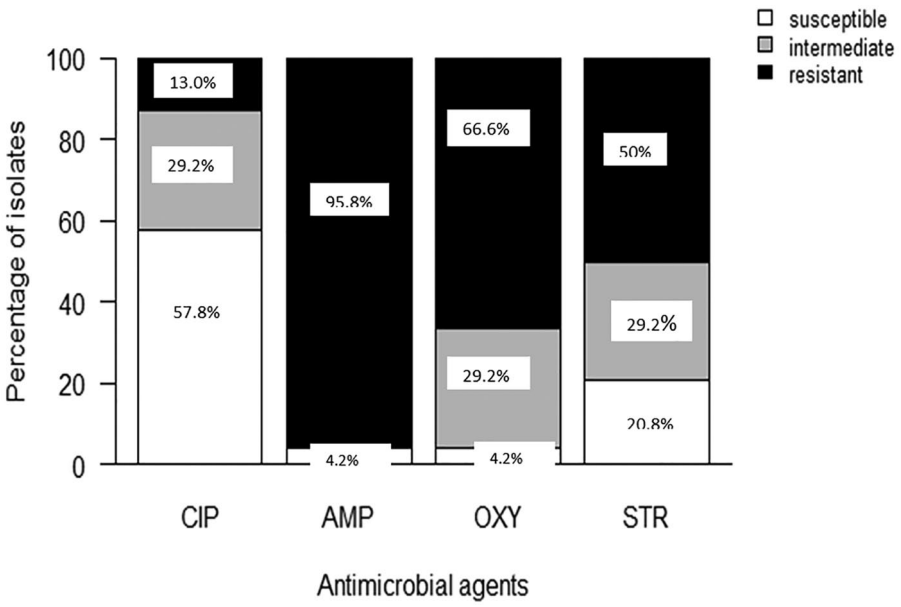


Figure 2. Multidrug resistance test results of bacterial isolates. Percentages of antimicrobial sensitivity of the isolates to the antimicrobials ciprofloxacin (CIP), ampicillin (AMP), oxytetracycline (OXY) and streptomycin (STR).

Providencia spp, *Serratia* spp. are known to show intrinsic resistance to colistin.[49–55] However, none of the colistin-resistant isolates obtained from this study belonged to any of these bacteria genera, suggesting that resistance in the isolates from our study may have been acquired.

The mechanism of acquiring resistance genes may occur by mutation or by the horizontal transfer of resistance genes via mobile genetic elements such as plasmids, integrons and transposon.[56] Among bacteria, plasmid-mediated transmission is the most common mechanism of horizontal gene transfer.[57] Plasmid-mediated resistance is the transfer of antibiotic resistance genes via plasmids.[58] Plasmids can be transferred between the same species of bacteria or different species via a mechanism known as conjugation.[59] Plasmid-mediated genes have the potential to transfer resistance, unlike intrinsic resistance that is caused by functional expression or mutation of chromosomal genes.[60] Since, the presence of plasmid-mediated genes was not investigated in this study, it could not be established whether resistant isolates had corresponding resistance genes.

All the colistin-resistant isolates were obtained from birds that were either omnivores or insectivores, yet, this may not be used as a risk factor since there were other omnivorous and insectivorous birds that did not harbour gram-negative bacteria,

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or colistin-resistant bacteria, suggesting that there may be a combination of factors that determine colonization of drug-resistant bacteria.

Since colistin is used in the treatment of multidrug resistant infections caused by gram-negative bacteria, all the colistin-resistant isolates were subjected to a multidrug resistance test. From the multidrug test, resistance to Ampicillin was demonstrated in 95.8% of the isolates. In a study of a wild migratory bird in Asia,[42] it was observed that colistin-resistant isolates were also resistant to Ampicillin. Bacteria genera such as *Enterobacter* and *Klebsiella* have been reported to show an intrinsic resistance to ampicillin.[61] Therefore, the high resistance to ampicillin may be intrinsic.

Our results are comparable to the results of a study that tested multidrug resistance of bacterial isolates from birds in the Brazilian Atlantic Forest to 5 antimicrobial agents (ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline) and found that 10% of their isolates showed a multidrug resistance pattern of ampicillin-tetracycline-chloramphenicol.[15] In this study, however, 50% of the isolates showed the multidrug resistance pattern of ampicillin-tetracycline-streptomycin. The presence of ampicillin, oxytetracycline and streptomycin in bacterial isolates corroborates findings on antimicrobial agent resistance to enterobacteriaceae from a variety of birds worldwide.[62–65]

Resistance to ciprofloxacin was the least observed in the multidrug resistance test. This antimicrobial agent belongs to the drug class quinolone which has had a restricted use in veterinary medicine since the 1990s after the rapid emergence of resistance to fluoroquinolones.[66] If our inference that acquisition of resistance is from livestock, then the above could explain the low resistance to ciprofloxacin. Moreover, in Ghana, some studies have reported the commonest quinolone used in animal production is enfloxacin and not ciprofloxacin.[31,67] Conversely, a study in Ghana investigating multidrug resistance in human isolates showed high levels of Ciprofloxacin resistance (>50%).[68] Ciprofloxacin is recommended for the treatment of urinary tract and blood stream infections in Ghana.[34]

This study was limited in some aspects for example, the 2 methods of bacteria identification were not adequate to confirm the species of bacteria. In addition, the number of antimicrobial agents used for the multidrug resistance test was few. Furthermore, colistin-resistant isolates were not examined for resistance genes, therefore, it could not be established whether resistant isolates actually had corresponding resistance genes. Nonetheless, this study has provided useful preliminary first-hand data on determination of antimicrobial resistance in wild birds from the Ankasa Conservation Area.

Conclusion

Despite the Ankasa Conservation Area being a protected area, antimicrobial resistance as well as multidrug resistance were observed in bacterial isolates sampled from birds inhabiting the area suggesting that although the area is protected, contaminations from external sources occur. This study has added on to the limited information on antimicrobial resistance in wild animals from protected areas in Ghana by providing data from which some observations/inferences were drawn. Firstly, resistance to colistin was observed, suggesting there is potential for the spread of resistant bacteria in the protected area. Secondly, it is possible that some of the gram-negative isolates that showed resistance to colistin may be pathogens that cause significant infection in humans. Thirdly, transmission of antimicrobial resistant bacteria may occur between wild animals, livestock and humans. However, the transmission routes remain unclear, though direct contacts with contaminated food, water and soil seem to be important routes for transmission. Finally, proximity of protected areas to human influenced habitats such as settlements and farmlands may pose more risk of acquiring antimicrobial-resistant bacteria, hence the need for buffer zones around protected areas.

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Supplemental material

Supplemental material for this article is available online.

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