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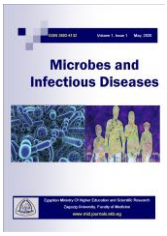

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Original article

Molecular detection of antibiotic resistance genes in avian pathogenic *Escherichia coli* isolated from poultry droppings in Bayelsa State, Nigeria

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ABSTRACT

Background: Avian pathogenic *Escherichia coli* (APEC) is becoming endemic in poultry farms worldwide and the incidence of antimicrobial resistance seemed to have followed the same trend. APEC is the causative agent of colibacillosis, which has caused economic losses in the poultry industry. In this study. **Methods:** We used multiplex PCR techniques to target antibiotic resistance genes (ARGs) from 10 APEC strains isolated from the droppings of chickens suffering from colibacillosis in the teaching and research farm of the Niger Delta University, Nigeria. **Results:** The result of the study showed that the APEC isolates possessed some ARGs coding for gentamicin, sulfonamide and cephalosporin, while other tested genes were absent. The prevalence of the detected genes were 80%, 30% and 20% for sull, blaCMY and aac(3)-IVgenes respectively. Most of the isolates had one ARG each. But about 30% of the isolates possessed two resistance genes each; sull and blaCMY in two isolates, and aac(3)-IV and sull in one isolate. The study established the presence of ARGs in the APEC isolated from the poultry droppings. **Conclusion:** The findings present a public health challenge because it could lead to transmission of antibiotic resistance to humans. We conclude that the surveillance of antibiotic resistance gene and pattern of APEC is essential for the control of diseases in both poultry and humans. We therefore recommend limited use of synthetic antibiotics in poultry.

Introduction

Avian pathogenic *Escherichia coli* (APEC) causes several infectious diseases in poultry collectively known as colibacillosis, which is an acute and systemic disease that has caused extensive mortality and economic losses in the poultry industry [1-4]. Colibacillosis is now regarded as an endemic disease of poultry worldwide [5]. **Maciel et al.** [3] reported mortality rate of 28.3% of 360 free-ranging chickens during an outbreak of colibacillosis in Brazil.

Antibiotics are drugs typically used for the control of infections both in humans and animal farming including poultry. In the poultry sector, antibiotics are used extensively as growth promoters and for the prevention and treatment of infectious diseases. The choice antibiotics used in poultry farming worldwide for the control of colibacillosis

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include β-lactams, sulphonamide, aminoglycosides and fluoroquinolones [6].

In recent years, due to the intensive and extensive use and abuse of antibiotics in animal farming especially in poultry, has resulted in the emergence of antimicrobial resistance (AMR), which in some cases have evolved into multidrug resistance (MDR) [3, 7-10]. Some of the adverse effects of excessive use of antibiotics in poultry include alteration in the gut microflora, occurrence of residual antibiotics in poultry products including meat and eggs, slow degradation of poultry wastes and attendant environmental impacts, all of which has led to the emergence of antimicrobial resistance among microbes both in humans and animals [4, 11,12]. The system of breeding has also been shown to influence the emergence of antibiotic resistance in poultry [13,14]. For instance, **Goudarztalejerdi et al.** [15] reported MDR among 81% of APEC and 73% of avian faecal *Escherichia coli* (AFEC) isolated from Iran. **Johar et al.** [8] reported that APEC is potentially transmissible to humans via the faecal–oral route, which could potentially cause various infections.The risk of transmission of ARGs in APEX to humans is increasing, not just through contaminated meat and eggs alone, but also through the use of chicken droppings as manure in farms[16]. Several authors have recognized the zoonotic potential of APEC [5, 6,11]. **Adefisoye et al.** [17] reported the presence of MDR strains of *Escherichia* coli *(E. coli*) and ARGs from treated municipal wastewater effluents in Eastern Cape, South Africa. **da Silva et al.** [18] reported that APEC exhibiting MDR can spread in the environment through the chicken droppings. Besides, excessive use of antibiotics in poultry can slow down the natural degradation of poultry droppings, potentially causing environmental menace (odour, aesthetic and disposal problems) and giving wider room for vectors acquisition and transmission of MDR strains, which could cause public heath challenges.

Antimicrobial resistance poses global public health challenges especially in the control of the burden of infectious diseases in humans and animals, and food safety [9, 10]. Food animals are commonly regarded as important vectors for acquisition and transmission of ARGs from the environment to humans [19]. Studies have shown that antibiotic resistance genes (ARGs) have been detected not only in domestic animals, but also wildlife especially those that range close to human dwellings [20-23]. **Ferreira et al.** [7] reported that AMR reduces therapeutic options and can increase the survival of the pathogen in the environment. More worrisome is the fact that it appears that the evolution of antibiotic resistance is outpacing the development of new antibiotics [17].

Antimicrobial resistance (AMR) and Multi-drug resistance (MDR) have been reported in APEC and AFEC, which has been linked to the presence of ARGs [24]. Antibiotic resistance genes could be acquired either by mutation or horizontal transfer [9]. The increase in antibiotic resistance among bacteria has been reported to be mostly due to mobile genetic elements such as plasmids, transposons, that can be spread quite easily among bacterial populations [9, 17, 21]. Genetic elements such as integrons and gene cassettes can quickly evolve and adapt through the acquisition and expression of novel genes [15]. *Escherichia coli* have been generally reported to have great capacity to acquire ARG through horizontal gene transfer. They have been reported to acquire genes coding for extended-spectrum β-lactamases (ESBL), and many other classes of antibiotics [21]. **Usman et al.** [4] linked the pathogenicity and AMR of APEC with the presence of virulence and AMR genes in poultry. **Seo et al.** [14] reported stains of *E. coli* with CMY-2-encoding genes from class 1 integrons with 10 different gene cassette arrangements. **Lentz et al.** [19] considered the presence of the blaSHV,blaTEM and blaCTX-M genes in the environment contributes to the dissemination of resistance to cephalosporins.

Notwithstanding the advancement in molecular biology, particularly the polymerase chain reaction (PCR) technology, information concerning the molecular basis of antimicrobial resistance in APEC is still scarce in Africa [2]. Hence, this study is focused on the use of multiplex PCR techniques for the molecular detection of ARGs from the droppings of birds suffering from colibacillosis in the Teaching and Research Farm of the Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria.

Materials and methods

Source of isolate

The ten isolates that was used for the study were obtained from the faeces of sick birds suspected to be suffering from colibacillosis at the Niger Delta University Teaching and Research Farm. *Escherichia coli* was isolated from the faecal matter

using MacConkey agar and eosin–methylene blue agar at the Microbiology Laboratory of the Niger Delta University. The pure cultures of the ten isolates were prepared on nutrient broth and stored in sterile McCartney bottle and incubated for 18hours at 35ºC before transporting them to the Nigerian Institute for Medical Research (NIMR) Yaba, Lagos for molecular analysis using multiplex PCR techniques. The DNA of the isolates were extracted using the boiling method as described by **Adefisoye et al.** [17] and were subsequently identified to be APEC through their possession of APEC virulent genes [25].

Extraction and determination of antibiotic resistant genes in APEC

The study was carried out to detect enzymes coding for antibiotic resistance. Multiplex PCR techniques targeting specific oligonucleotide primers **(Table 1)** was used for the identification of the presence of resistant genes; streptomycin[aadA1], gentamycin[aac(3)-IV], sulfonamides[sull], beta-lactams [blaSHV, blaCMY, blaTEM]and erythromycin[ere(A)]. The multiplex PCR used for the study was modified from **Adefisoye et al.** [17] and **Momtazet al.** [27]. It was performed in a 20µl reaction mixture containing 1X Blend Master mix buffer (Solis Biodyne) containing 1.5 mM MgCl2, 200µM of each deoxynucleotide triphosphates (dNTP) (Solis Biodyne), 20 pMol of each primer (Stab Vida, Portugal), 2 units of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 2µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture.Thermal cycling was conducted in a Peltier Thermal Cycler PTC 200 (MJ Research) for an initial denaturation of 95°C for 15 minutes followed by 30 amplification cycles of 30 seconds at 95°C; 30 seconds at 60°C and 1 minute 30 seconds at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis 100V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker.

Table 1. Resistance gene primers for streptomycin, gentamycin, sulfonamides, beta-lactams, erythromycin

Antibiotic	Enzyme	Gene	Primer sequence	Size (bp)	Reference
Streptomycin	Adenylyl transferases (*aadA1*)	*aadA1*F	TATCCAGCTAAGCGCGAACT	447	[27, 26]
		*aadA1*R	ATTTGCCGACTACCTTGGTC		
Gentamicin	Aminoglycoside acetyltransferases (*aac*(*3*)-*IV*)	*aac*(*3*)-*IV*F	CTTCAGGATGGCAAGTTGGT	286	[27, 26]
		aac(*3*)-*IV*R	TCATCTCGTTCTCCGCTCAT		
Sulfonamide	Dihydropteroate synthase (*sul1*)	*sul1*F	TTCGGCATTCTGAATCTCAC	822	[27, 26]
		sul1R	ATGATCTAACCCTCGGTCTC		
Beta-lactams	*β*-lactamase encoding penicillin resistance *(bla*$_{SHV}$*)*	*bla*$_{SHV}$F	TCGCCTGTGTATTATCTCCC	768	[27, 26]
		bla$_{SHVR}$	CGCAGATAAATCACCACAATG		
	β-lactamase encoding ampicillin resistance *(bla*$_{TEM}$*)*	*bla* $_{TEM}$F	ATT CTT GAA GAC GAA AGG GC	1150	[28, 29]
		bla $_{TEM}$R	A CG CTC AGT GGA ACG AAA AC		
	β-lactamase encoding cephalosporin resistance (*bla*$_{CMY}$)	*bla*$_{CMY}$F	TGGCCAGAACTGACAGGCAAA	462	[27, 26]
		bla$_{CMY}$R	TTTCTCCTGAACGTGGCTGGC		
Erythromycin	esterase (*ere*(A))	*ere*(A)F	GCCGGTGCTCATGAACTTGAG	419	[27, 27]
		ere(A)R	CGACTCTATTCGATCAGAGGC		

Note: F = forward primer, R =reverse primer

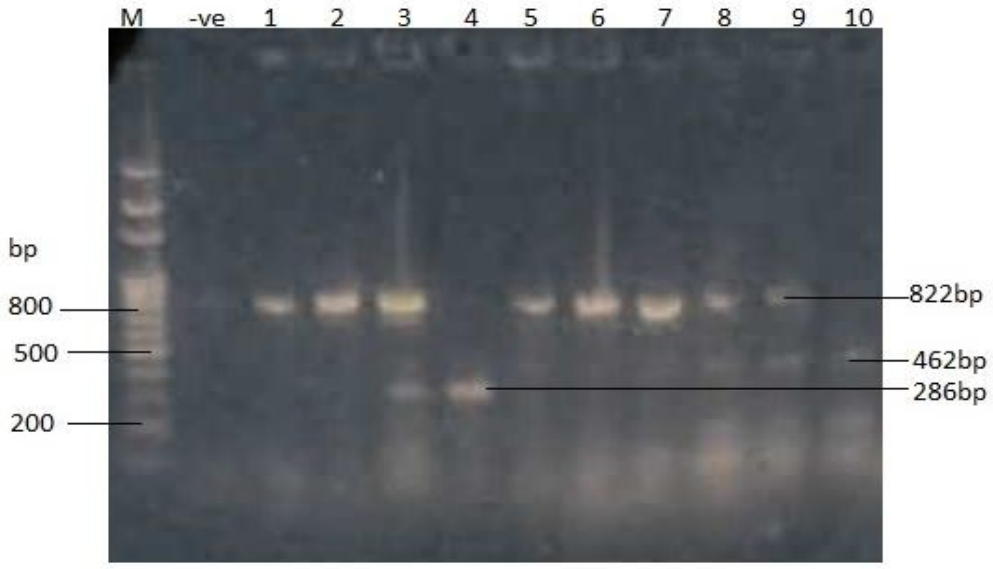
Results

The result of the study showed that the APEC isolates used for the study possesses some ARGs **(Figure 1)** coding for gentamicin, sulfonamide and cephalosporin, while other tested genes were absent **(Table 2).** The results show that the sull gene have been detected in eight out of the ten isolates, have prevalence of 80% was the most prevalent, followed by blaCMY and aac(3)-IV, which had a prevalence of 30% and 20% respectively. Most of the isolates had one ARG each. But about 30% of the isolates possessed two resistance genes each; sull and blaCMY in two isolates, and aac(3)-IV and sull in one isolate. There was no instance of more than two resistance genes detected in any of the isolates, hence by the definition criteria of the presence of three or more ARGs in a strain for it to be classified as MDR [27, 30], none of the isolates can therefore be said to be MDR. Perhaps, if larger number of isolates or ARGs were tested, there might be cases of MDR.

Table 2. Incidence of antibiotic resistant genes among the isolates.

Antibiotics	**Resistance Gene**	**% Gene presence**
Streptomycin	*aadA1*	0
Gentamicin	*aac*(*3*)*-IV*	20
Sulfonamide	*Sull*	80
Penicillin	bla_{SHV}	0
Ampicillin	bla_{TEM}	0
Cephalosporin	bla_{CMY}	30
Erythromycin	*ere*(A)	0

Figure 1. PCR result for the multiplex PCR identification of the resistant genes.



Discussion

The study show that APEC strains isolated from the droppings of chicken in the Research and Teaching Farm of the Niger Delta University possesses ARGs encoding for Gentamicin, Sulfonamide and Cephalosporin, thus, establishing AMR. This present a public health challenge because it could lead to transmission of antibiotic resistance to humans through various means such as contact or through food consumption [19]. Besides, chicken wastesare commonly used as manure[16], which therefore have the tendency of transmitting ARGs to other organisms including commensals in wildlife and the general environment [9, 10]. For this reason, poultry are sometimes regarded as reservoir for ARGs[19].

Other authors have similarly reported the presence of several ARGs in poultry especially those infected with APEC or suffering from colibacillosis. **Hossain et al.** [31] reported 56.0% of sull gene among other ARGs from *E. coli* isolated from frozen chicken displayed for sale in supermarkets in Bangladesh. **Rossato et al.** [32] reported the highest resistance to sulphonamide among other antibiotics by *E. coli* isolated from poultry feed and ingredients. **Rahman et al.** [24] studied the prevalence ARGs in broiler chicken APEC isolates, and reported 44.16% for sul1 and 25.38% for aac-3-IV among other ARGs. They also reported instances of MDR. **Van et al.** [30] detected the sul1 gene from *E. coli* isolated from chicken.

Cephalosporin is an ESBL and many authors have observed resistance genes to this group of antibiotics among poultry [2, 9, 14, 33]. **Messele et al.** [26]observed the presence of AMR genes in chicken meatwith cases of MDR. They reported prevalence of ARG of blaCMYto be 65.1% and sul1to be54.0%, while 39.7% % exhibited MDR. **Shin et al.** [33] found that blaCMY genes have the tendency to be transferablevia plasmids. **Hansen et al.** [34]detected the blaCMY-2 genes from clinical and commensal strains of *E. coli* isolated from humans, chickens and dogs. **Rybak et al.** [35]detected cephalosporin resistance gene from *E. coli* isolated from the faeces of free-ranging birds in human environment. **da Silva et al.[**18] reported 87% of APEC isolatesproduced ESBL, which were suspected of having zoonotic potential. **Usman et al.** [4]reported resistance to gentamicin among other antibiotics in APEC isolated from chickens in Pakistan. Other authors have similarly reported resistance to gentamicin among other antibiotics **Maciel et al.** [3]. **Chalmers et al.** 36]detected blaCMY, aac(3)-VI genes in *E. coli* isolates from chickens suffering from colibacillosis in Québec, Canada.

Conclusion

Poultry is one of the most important sources of healthy animal protein in the world. Antibiotic play a major role is preventing and treating microbial infections in poultry farms. Unfortunately, the excessive use of antibiotics in animal production has led to the increasing problems of antibiotic resistance. Meanwhile, poultry droppings are increasingly being used as manure. This study sampled and analysed for ARGs in the droppings of poultry suffering from colibacillosis in the research and teaching farm of the Niger Delta University. We detected the presence of genes responsible resistance to gentamicin, sulfonamide and cephalosporin and could therefore speculate that poultry droppings are possible routes for the transmission of ARGs.We therefore recommend that the use of synthetic antibiotics in poultry should be restricted, while research attention should focus on their replacement with alternatives such as probiotics and phytobiotics.

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Conflict of interest

Not declared.

Funding

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