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Molecular determination of virulent genes from avian pathogenic *Escherichia coli* isolated from poultry farm droppings in Bayelsa State, Nigeria

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ABSTRACT

Background: Colibacillosis caused by avian pathogenic *Escherichia coli* (APEC), which is an extraintestinal pathogenic *E. coli* (ExPEC), is the commonest bacterial disease of poultry, which has led to severe economic losses. **Methods:** In this study, we isolated ten suspected strains of *Escherichia coli* (*E. coli*) from the droppings of birds suffering from colibacillosis at the teaching and research farm of the Niger Delta University, Wilberforce Island, Nigeria. Eight virulence associated genes (VAGs) were analysed for, using multiplex PCR techniques. **Results:** Only four (Isolates 1, 5, 6 and 8) were positive for the tested virulent genes. The iron uptake gene *iutA*, was predominant, being present in all the four isolates (1, 5, 6 and 8). Another iron uptake gene *iucD*, was detected only in isolates 1 and 5, while outer membrane protein gene *ompT*, was detected in isolates 1 and 8 only. The toxin gene *hlyF*, was only detected in isolate 8. In all, one VAG was detected in isolate 6 (*iutA*), two VAGs in isolate 5 (*iutA*, *iucD*) and three each in isolate 1 (*iutA*, *iucD*, *ompT*) and isolate 8 (*iutA*, *ompT*, *hlyF*). When isolate 1 was inoculated in a healthy chicken, it exhibited symptoms of colibacillosis with visible lesions. **Conclusions:** The results confirm the importance of VAGs in the pathogenesis of APEC.

Introduction

Avian pathogenic *Escherichia coli* (APEC) is a strain of *E. coli* that causes diseases primarily in poultry leading to economic losses [1]. The pathogen has been implicated in several infectious diseases of birds, of which colibacillosis is by far the most common bacterial disease [2, 3]. Nakazato et al. [1] reviewed several diseases associated with APEC including colisepticaemia (colibacillosis), inflammation of the oviduct, salpingitis, peritonitis, cellulitis and swollen head syndrome.

Colisepticemia caused by APEC often facilitates other infections such as viral (bronchitis virus, Newcastle virus) and other bacterial (*Mycoplasma*) infection of the upper respiratory system [4]. The infectious often results in yield loss and ultimately death of chickens. Bhattarai et al. [3] estimated economic losses linked to colibacillosis including causing up to 20% mortality, reduction in meat production by about 2% live weight loss and 2.7% reduction in feed conversion ratio, up to 20%

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reduction in egg production and up to 43% carcass rejection by customers.

Janßen et al. [5] analysed for 17 virulence-associated genes (VAG) in APEC isolated from the internal organs of chickens that died from colibacillosis, among which *iucD*, *fimC*, *tsh*, *stx2f* and *hlyE* were used to identify APEC. They found that a combination of *tsh*, *iucD*, *fimC*, *fyuA* and *irp2* genes rendered the strains predominantly virulent. **Bhattarai et al.** [3] reported that the major VAGs predominant among APEC involved in colibacillosis were *traT*, *iutA*, *iss*, *hlyF*, *iroN*, *fimH*, *ompT*, *iucD*, *cvi/cva*, *sit chro.*, *tsh*, *kpsMTII* and *aerJ*.

Pathogenesis are somewhat related to virulence. Although, *E. coli* is a normal flora in the guts of humans, other mammals and birds, extraintestinal pathogenic *E. coli* (ExPEC) like APEC exhibit enhanced ability to cause infectious diseases even beyond the gut of chickens [6], hence they are referred to as facultative pathogens [7]. The survival of *E. coli* beyond the gut of birds is facilitated by the presence of several virulence factors (VF) which are coded by virulence genes [8]. **Dissanayake et al.** [9] reported that ExPEC enters through diverse routes particularly respiratory and genital tracts causing various extra intestinal illnesses collectively known as colibacillosis [8]. APEC have been reported to be related to extraintestinal human strains such as uropathogenic *E. coli* (UPEC), sepsis-associated *E. coli* (SEPEC) and neonatal meningitis-causing *E. coli* (NMEC) [1, 7, 10, 11]. **Bhattarai et al.** [3] reported that human ExPECs share major 10 virulence genes with APEC including *iucC*, *iucD*, *cvaA*, *cvaB*, *cvaC*, *iutA*, *etsA*, *ompT*, *hlyF* and *cvi* suggesting a probable zoonotic transmission of APEC from birds to humans. Several other authors have mentioned the zoonotic potential of APEC [1, 9, 11-16]. **Olsen et al.** [12] detected virulence genes in APEC including *iss*, *ompT*, *hlyF*, *iutA*, *traT*, *eitABC*, *sitABCD*, *iroBCDEN*, and *lucABCD*, several of which are found in with human ExPEC.

APEC virulence gene contribute to pathogenicity of the strain through production of toxins, iron uptake mechanisms, adhesion, invasion, immune suppression, resistance to host's serum and bacterial survival [1, 13], which are typically encoded on plasmids, pathogenicity islands (PAIs), transposons, bacteriophage and other mobile genetic elements [10, 7, 17]. For instance, **Singh et al.** [18]

detected the 'tsh' gene in *E. coli* isolates that correlated positively with pathogenicity *in-vivo*. The *iss* gene increases resistance to serum [19]. **Hussain et al.** [13] reported the most prevalent virulence genes associated with multidrug resistant APEC to be *iroN*, *papC*, *iucD*, *fimC*, *fimH*, *traT*, *irp2*, and *iutA*. **Ievy et al.** [20] reported multidrug resistance of APEC possessing three virulence genes (*papC*, *iucD* and *fimC*) for the first time in Bangladesh. Other authors have also reported multidrug resistance (MDR) linked to the virulent and drug resistance genes of APEC [17]. **Lee et al.** [21] reported that tetracycline resistance in *E. coli* is associated with the presence of virulent genes particularly increased serum survival (*iss*), iron uptake (*iroN*) and temperature-sensitive haemagglutinin (Tsh) genes.

Based on detection frequency, subcellular localization and protein uniformity, some of the VAGs including *iroN*, *iss*, *ompT*, *iutA*, *traT* and *etsC* has been suggested as suitable vaccine candidates [12]. **Kathayat et al.** [11] suggests that anti-virulence inhibitors can be explored as alternative measure for the control of APEC in poultry especially because the strains are unlikely to develop resistance to these inhibitors. Also, some virulence genes that do not play a role in pathogenesis of APEC and its drug resistance patterns can be explored for the development of countermeasures such as vaccines and inhibitors.

Authors have used a combination of three or more VAGs viz; *iroN*, *ompT*, *iucD*, *iss*, *ompT*, *hlyF* and *iutA* to classify strains of *E. coli* as APEC [8, 22]. **Aslam et al.** [14] defined ExPEC with the presence of at least two of the following VAGs; *papA/papC*, *iutA*, *sfa* and *kpsMT II*. However, **Johnson et al.** [23] observed that the APEC plasmid used as defining trait of APEC are prevalent both in diseased birds and in the caecum of healthy birds. They suggested sequence types (STs) especially ST23, ST131, ST117, ST355 and ST428 which dominated infected birds but rare in the caecum of healthy birds. **Dissanayake et al.** [9] found that a combination of four genes (*ompT*, *iroN*, *hlyF* and *papC*) adequately predicted APEC and also reported four STs (ST10, ST117, ST48 and ST2016) contained APEC isolates. Hence, the aim of this study was to characterize APEC strains isolated from the droppings of poultry suspected to be suffering from colibacillosis for the presence of VAGs.

Material and methods

Source of isolate

The isolate used for the study was obtained from the droppings of sick birds suspected to be suffering from colibacillosis. *Escherichia coli* was isolated from the droppings using MacConkey agar and eosin–methylene blue agar at the Microbiology Laboratory of the Niger Delta University. Ten pure cultures of the isolates were prepared on Nutrient broth and stored in sterile McCartney bottle and incubated for 18 hours at 35°C before transporting them to the Nigerian Institute for Medical Research (NIMR) Yaba, Lagos for molecular analysis.

DNA extraction and detection of virulence genes

DNA was extracted on overnight culture of the bacteria using the Jena Bioscience DNA purification kit according to the manufacturer's instructions (Jena Bioscience GmbH, Germany). Virulent genes of the isolates were identified using a multiplex PCR technique. The ten isolates were screened for eight virulent genes which include *iutA*, *iss*, *ompT*, *iroN*, *hlyF*, *vat*, *papC* and *iucD* using their respective primers (Table 1). In order to test for the virulent genes, two multiplex PCR cycles were carried out as described below. The first multiplex PCR involved identifying the presence of APEC virulent genes; *iroN*, *ompT*, *hlyF*, *iutA* and *iucD*. The multiplex PCR was done in a 20µl reaction mixture containing 1X Blend Master mix buffer (Solis Biodyne), 1.5 mM MgCl₂, 200µM of each deoxynucleotide

triphosphates (dNTP) (Solis Biodyne), 2 units of hot FIREPol DNA polymerase (Solis Biodyne), 20 pMol of each primer (Stab Vida, Portugal), proofreading Enzyme, 2µl of the extracted DNA, while sterile distilled water was used to make up the reaction mixture.

The second multiplex PCR involved identifying the presence of the APEC virulent genes *iss*, *vat*, and *papC*. The multiplex PCR was done in a 20µl reaction mixture comprising of 1X Blend Master mix buffer (Solis Biodyne), 200µM of each dNTP (Solis Biodyne), 1.5mM MgCl₂, 20 pMol of each primer (Stab Vida, Portugal), 2 unit of hot FIREPol DNA polymerase (Solis Biodyne), proofreading Enzyme, 2µl of the extracted DNA, while sterile distilled water was added to make up the reaction mixture.

Thermal cycling was conducted using the MJ Research Peltier Thermal Cycler, PTC 200 (GMI, Inc) for an initial denaturation of 95°C for 15 minutes followed by 30 amplification cycles of 30 seconds at 95°C, 30 seconds at 56°C and 1 minute at 72°C. This step was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 1.5% agarose gel electrophoresis that was carried out at 100V for 1 hour 30 minutes, which was followed by DNA bands visualization using ethidium bromide staining. A 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker.

Table 1. Virulence gene primers for APEC.

Gene	Description	Primer sequence	Size (bp)	Reference
iutA	Iron uptake	F:GGCTGGACATCATGGGAACTGG R: CGTCGGGAACGGGTAGAATCG	302	[24]
iucD	Iron uptake	F:ACAAAAAGTTCTATCGCTTCC R: CCTGATCCAGCTGATGCTC	714	[21, 25]
iroN	iron uptake	F: AATCCGGCAAAGAGACGAACCGCCT R: GTTCGGGCAACCCCTGCTTTGACTTT	553	[24]
Iss	serum survival	F: ATCACATAGGATTCTGCCG R: CAGCGGAGTATAGATGCCA	309	21, 25]
ompT	Outer membrane protein	F:TCATCCCGGAAGCCTCCCTCACTACTAT R: TAGCGTTTGGCTGCACTGGCTTCTGATAC	496	[23, 24]
hlyF	Toxin	F:GGCCACAGTCGTTTAGGGTGCTTACC R: GGCGGTTTAGGCATTCCGATACTCAG	450	[23, 24]
vat	Toxin	F: TCCTGGGACATAATGGTCAG R: GTGTCAGAACGGAATTGT	981	[25]
papC	Adhesins for attachment	F: TGATATCACGCAGTCAGTAGC R: CCGGCCATATTCACATAA	501	[21, 25]

F=forward primer, R=Reverse primer

Results and discussion

Of the ten isolates (isolates 1-10), only four (Isolates 1, 5, 6 and 8) were positive for the tested virulent genes, hence, 40% of the isolates tested positive for APEC genes. Among the eight VAG, tested, only 4 (*iutA*, *iucD*, *ompT*, *hlyF*) was detected in all the isolates (**Figure 1**). The genes that were not detected in any of the isolates were *iss*, *vat*, *iroN* and *papC* (**Figure 2**). The iron uptake gene *iutA*, was present in all the four isolates (1, 5, 6 and 8), i.e. 100% presence in all the positive isolates. Another iron uptake gene *iucD*, was detected only in isolates 1 and 5, thus representing 50%. The outer membrane protein gene *ompT*, was detected in isolates 1 and 8 only, which therefore represented 50% among the positive isolates. The toxin gene *hlyF*, was only detected in isolate 8, thus representing 25% of the positive isolates. In all, one VAG was detected in isolate 6 (*iutA*), two VAGs in Isolate 5 (*iutA*, *iucD*) and three each in isolate 1 (*iutA*, *iucD*, *ompT*) and isolate 8 (*iutA*, *ompT*, *hlyF*). When isolate 1 was inoculated in a healthy chicken, it exhibited symptoms of colibacillosis with visible lesions. The results show that iron uptake gene (*iutA*) was predominant among the isolates.

Using multiplex PCR technique, **Abu El Hammed et al.** [26] determined the prevalence of APEC in *E. coli* isolates based on the detection of 8 VAGs viz; *iss*, *astA*, *irp2*, *iucD*, *papC*, *tsh*, *cva/cvi* and *vat*. **Bhattarai et al.** [3] analysed for 57 VAGs from four types of chickens infected by colibacillosis in Nepal, and found that the top 5 VAGs are *fimH*, *iss*, *traT*, *sit chro* and *ironEC*. Using multiplex PCR techniques, **Johnson et al.**

[24] screened 124 avian *E. coli* isolates of known pathogenicity for 44 VAGs and demonstrated that five genes (*iutA*, *iss*, *hlyF*, *ompT* and *iroN*) carried by plasmids were the most significantly associated with highly pathogenic APEC strains. **Hossain et al.** [27] used four virulence genes, *iutA*, *hlyF*, *iroN*, and *iss* detected by PCR analysis to characterize APEC in Bangladesh. Other authors have also used the presence of virulent genes to identify APEC linked to colibacillosis in poultry [28 - 31].

In our study, isolate 1, in which three VAGs (*iutA*, *iucD*, *ompT*) were detected, was able to induce colibacillosis in inoculated healthy chicken. This could either mean that these three genes were enough to induce the disease or more VAGs were probably present, which were not analyzed for. The relatively small number of isolates studied is a limitation of this work. Notwithstanding, our study, confirms the detection of markers of APEC in the birds suffering from colibacillosis and shows that VAGs play prominent roles in their pathogenicity. Similarly, other authors have reported VAGs contributing to invasiveness and pathogenicity of other bacteria. For instance, **Oladapo et al.** [32] detected *hil A*, *iro B* and *stn* genes of *Salmonella enterica* serovar typhimurium from non-ripened cheese, which are associated with pathogenicity of the organism. Likewise, **Olusola-Makinde et al.** [33] demonstrated the presence of virulence factors in *Proteus penneri* and *Bacillus cereus* responsible for haemolytic activities of the bacteria. These studies linked the contribution of genetic elements to bacterial pathogenicity.

Figure 1. Result for the multiplex PCR identification of the virulent genes *ompT*, *hlyF*, *iutA* and *iucD*.

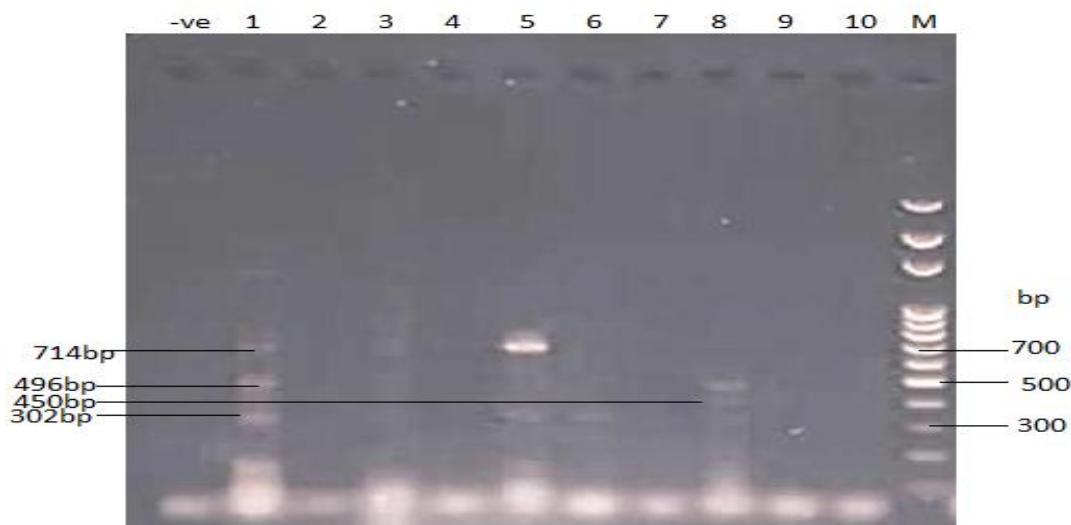
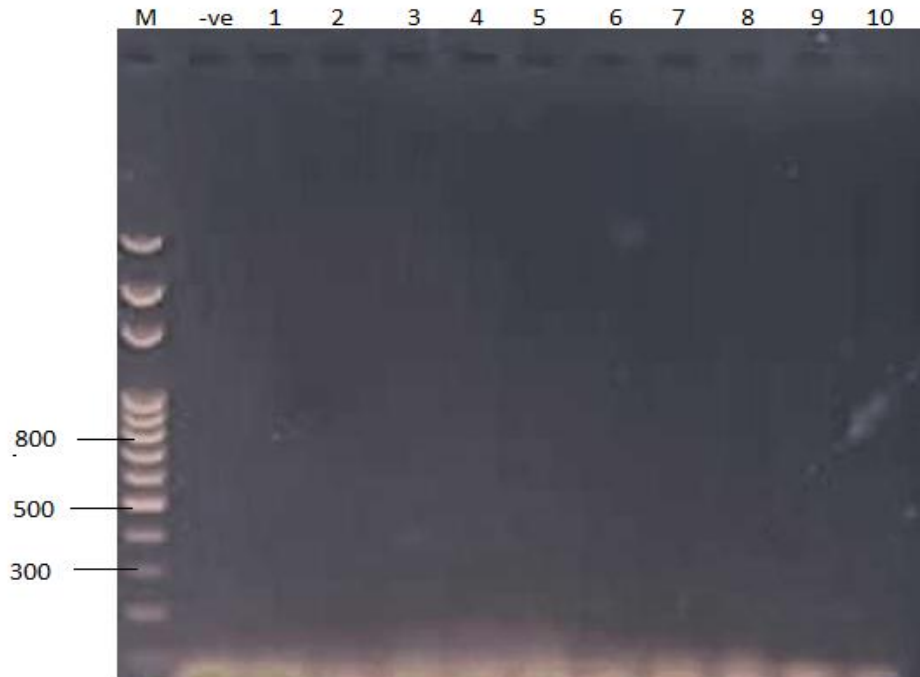


Figure 2. Result for the multiplex PCR identification of the virulent genes *iss*, *vat*, and *papC*.

Conclusion

Ten strains of *E. coli* that was isolated from the droppings of chickens suffering from colibacillosis was analysed for the presence of 8 VAGs using multiplex PCR techniques. The study detected the presence of four VAGs (*iutA*, *iucD*, *ompT*, *hlyF*) that characterized APEC. Isolate 1, which expressed three of the genes (*iutA*, *iucD*, *ompT*), induced colibacillosis in healthy birds when inoculated. The iron uptake gene, *iutA* was detected in all the positive isolates. The study therefore confirms the importance of VAGs in the pathogenesis of APEC and their use as markers for its determination.

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Conflicts of interest

Not declared.

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