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AVIAN PATHOGENIC *ESCHERICHIA COLI* IN DISEASED CHICKENS FROM COMMERCIAL FARMS IN NORTHWEST, NIGERIA

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ABSTRACT

Avian colibacillosis, which is caused by avian pathogenic *Escherichia coli* (APEC), is a major bacterial disease that affects birds of all ages worldwide, causing significant economic losses. APEC manifests in several clinical forms, including cellulitis, enteritis and several extra-intestinal diseases such as Colibacillosis, Airsacculitis, Omphalitis, Acute septicemia, Salpingitis. This study was carried out to investigate avian pathogenic *Escherichia coli* in diseased chickens

Introduction

Escherichia coli capable of causing localized or systemic extraintestinal infections in Chickens, turkeys, geese, ducks, and other species of birds is known as APEC (Circella *et al.*, 2012; Nolan *et al.*, 2013; Guabiraba & Schouler, 2015). Avian pathogenic *E. coli* can cause infections in all age groups of birds (Nolan *et al.*, 2013; Guabiraba & Schouler, 2015), acting as a primary (Vandekerchove *et al.*, 2004b) or secondary pathogen (Kaper *et al.*, 2004; Kabir, 2010; Nolan *et al.*, 2013; Guabiraba & Schouler, 2015). Although APEC is present in the intestinal microbiota, the main route of entry into birds is through the respiratory system via inhalation of APEC-contaminated aerosols. The bacteria can then translocate from the respiratory tract (air sac and lungs) to the bloodstream and

from commercial farms in Sokoto and Kebbi States. A total of 450 cloacal swabs were collected, screened and analyzed using standard culture, isolation biochemical characterization, and molecular techniques. Three hundred and seventy 370 (82.2%) were positive to culture and isolation while one hundred and four 104 (23.1%) were positive to biochemical tests. Following polymerase chain reaction techniques only one hundred and one 101 (22.4%) isolates were confirmed *Escherichia coli*. Pathotyping of all the confirmed *Escherichia coli* isolates were carried out to determine the virulence types using the minimum number of virulence-associated genes (VGs) that could identify APEC (*iutA*, *iss*, *ompT*, *iroN* and *hlyF*). All isolates were found to be APEC. Among 101 confirmed *Escherichia coli* isolates, none of the strains contained one virulence gene, no strains contained only two virulence genes, four (4) strains contained three virulence genes, and 30 strains contained four virulence genes. All the five virulence genes *iss, ompT, hlyF*, and *iroN* were detected in 67 strains of the confirmed *Escherichia coli* isolates. Therefore, all the confirmed 101 (100%) *Escherichia coli* isolates have a minimum of either three (3), four (4), or five (5) VGs (*iutA*, *iss*, *ompT*, *iroN* and *hlyF*) which are the minimal predictors of APEC, are therefore confirmed to be all APEC pathotypes. Based on the study findings, avian pathogenic *Escherichia coli* in diarrhea chickens from commercial farms in the study areas has been established, therefore commercial farms in the study areas should implement stricter biosecurity practices to reduce the spread of *E. coli* and introducing vaccines specifically designed to target avian pathogenic *E. coli* (APEC) strains could significantly lower infection rates and reduce reliance on medications.

Keyword: Avian, Pathogenic, *Escherichia Coli,* Diseased, Chicken.

other internal organs (Delicato *et al.*, 2003; Dissanayake *et al.*, 2014). Systemic infection can also occur after *E. coli* in the cloaca or the intestinal tract gain access to the bloodstream (Landman & Cornelissen, 2006; Kabir, 2010).

The pathogenicity of APEC strains is multifactorial, based on the presence and expression of different groups of VGs (Won *et al.*, 2009). Globally, the economic, public health, and animal welfare significance of avian colibacillosis, has led to several studies from the United States of America aiming to define APEC and to identify pathogenicity mechanisms (Jeffrey *et al.*, 1999; Norton *et al.*, 2000; Kariyawasam & Nolan, 2009). Studies have identified a large number of VGs in *E. coli* cultured from birds with avian colibacillosis (Antão *et al.*, 2008; Johnson *et al.*, 2008b; Wang *et al.*, 2010; Dissanayake *et al.*, 2014;

Guabiraba & Schouler, 2015; Paixao *et al.*, 2016). The involvement and relevance of these VGs in the pathogenicity of avian colibacillosis is still poorly understood and the majority of studies on APEC and APEC-associated VGs are descriptive (Dozois *et al.*, 1995; Jeffrey *et al.*, 1999; Norton *et al.*, 2000; Zhao *et al.*, 2009; Obeng *et al.*, 2012). Few *in vivo* experimental studies have enhanced the understanding of the APEC pathogenicity mechanisms (Ask *et al.*, 2006; Antão *et al.*, 2008; Musa *et al.*, 2009). However, no single or specific set of VGs has been systematically linked with APEC pathogenicity. It is very rare to find the same set of VGs in all APEC, in the same study and/or different studies (Rodriguez-Siek *et al.*, 2005a; Zhao *et al.*, 2005; Kawamura-Sato *et al.*, 2010; Kemmett *et al.*, 2013; Paixao *et al.*, 2016). Nevertheless, APEC harbors several VGs that enable the bacteria to invade, colonize, evade the immune system, and cause avian colibacillosis (Horn *et al.*, 2012; Pires-dos-Santos *et al.*, 2013; Guastalli *et al.*, 2013; Kemmett *et al.*, 2014). The genetic diversity displayed by APEC makes it very challenging to diagnose, prevent, and treat avian colibacillosis. Many *E. coli* strains are not virulent and a fast, accurate diagnostic test to distinguish whether an avian *E. coli* isolate is pathogenic or non-pathogenic, is required. However, several studies have suggested that the prevalence of certain VGs among *E. coli* isolates obtained from Chickens with colibacillosis was useful in identifying and characterizing APEC and distinguish it from NON-APEC (Ewers *et al.*, 2005; Rodriguez-Siek *et al.*, 2005a; Moulin-Schouleur *et al.*, 2007; Johnson *et al.*, 2008b). A study by Ewers *et al.* (2005) suggested that eight VGs: P-fimbriae (*papC*); aerobactin (*iucD*); iron-repressible protein (*irp2*); temperature-sensitive hemagglutinin (*tsh*); vacuolating autotransporter toxin (*vat*); enteroaggregative toxin (*astA*); increased serum survival protein (*iss*) and colicin V plasmid operon genes (*cva/cvi*) contributed to the pathogenicity of APEC. The authors proposed that the presence of four of these eight VGs could identify APEC and differentiate between APEC and non-APEC. However, only 14 APEC isolates were included in this study reducing the strength of their conclusions. In a study by Kemmet *et al*. (2013) a larger number of faecal *E. coli* isolates (n = 3,360) obtained from apparently healthy Chickens and 324 *E. coli* isolates from birds with colibacillosis were screened for the presence of ten VGs (*astA*, *iss*, *irp2*, *iucD*, *papC*, *tsh*, *vat*, *cvi*, *sitA* and *ibeA*). The authors reported that 24% of the non-APEC isolated from one-day-old healthy Chickens harbored more than five of these VGs in comparison with 1% of non-APEC sourced from Chickens at slaughter. They found that *irp2, papC, iucD*, *cvi*, *sitA,* and *ibeA* genes were significantly associated with APEC. Rodriguez-Siek *et al.* (2005a) investigated the prevalence of 38 APEC-associated VGs among 451 APEC and 104 non-APEC isolates obtained from birds in the USA. They reported that the majority of APEC isolates harbored *ompT*, several iron acquisition genes (*iroN*, *iutA*, *sitA*, *fyuA,* and *irp2*), and other VGs genes that are carried in the APEC plasmid pTJ100 (*cvaC*, *iss*, *tsh*, *iroN*, *iutA,* and *sitA*).

Another study in the USA conducted by Johnson *et al.* (2008b) aimed to identify a minimum number of VGs that could identify APEC. In the study, 124 *E. coli* isolates of known pathogenicity underwent extensive genotyping and were screened for the presence of 46 VGs. The author identified five VGs (*iutA*, *iss*, *ompT*, *iroN,* and *hlyF*) that were significantly associated with APEC strains. Johnson *et al.* (2008b) developed a pentaplex-PCR, targeting these five VGs, and subsequently screened 994 *E. coli* (794 APEC and 200 non-APEC) to validate the selection of the five VGs. The results showed that highly pathogenic APEC strains harbored a minimum number of 5 VGs while VGs of 4 and 3 were found among medium and low pathogenic APEC strains, respectively. The number of VGs in non-APEC isolates was 1-2. Subsequently, studies have used the pentaplex-PCR described by Johnson *et al.* (2008b) and confirmed that it is a rapid diagnostic tool to differentiate between APEC and NON-APEC (Hussein *et al.*, 2013; Dissanayake *et al.*, 2014; De Oliveira *et al.*, 2015). Despite the geographical variations in the studies, Kobayashi *et al*. (2011) (68% – 72%), Hussein *et al*. (2013) (90% – 94%) and de Oliveira *et al*. (2015) (82% – 95%) reported similar frequencies of the five VGs as Johnson *et.al.* (2008b) (78% – 85%) among APEC isolates cultured from lesions of birds affected with colibacillosis. However, Dissanayake *et al.* (2014) found that only 25% of the *E. coli* isolates (14 of 55), were cultured from the lesions of birds with colibacillosis in Sri Lanka, and harbored the five VGs. These authors identified four VGs (*sitA*, *ompT*, *hlyF,* and *iroN*) that were significantly associated with APEC in their region. These four genes were possessed by 54.5% (30 of 55) of the APEC isolates in Sri Lanka.

A study by Schouler *et al*. (2012) examined 1,491 *E. coli* (1,307 APEC and 184 NON-APEC). They identified 13 VGs (*sitA, F1, iutA, tsh, frzorf4, tkt1, aec4*, P(F11), *aec26, neuC,sfa-focDE, cdt,* and *eae*) more frequently found in APEC and four different VG associations (A [*iutA*+, P(F11)+], B [*iutA*+, P(F11)–, *frzorf4*+], C [*iutA*+, P(F11)–, *frzorf4*–, O78+] and D [*iutA*–, *sitA*+, *aec26*+]). These four different VG associations identified 70.2% (247 of 352) of the pathogenic strains, based on a lethality test. In a more recent study, in Brazil, de Oliveria *et al.* (2015) investigated the association between the APEC VGs proposed by Johnson *et al.* (2008b) and the pathogenicity of the APEC strains. In partial agreement with Johnson *et al.* (2008b), they identified a positive association between the number of VGs and the pathogenicity score of avian colibacillosis. Based on the genetic criteria for the pathogenicity, isolates containing at least three virulence genes were considered the APEC strains, and isolates containing less than three virulence genes were considered the avian nonpathogenic *Escherichia coli* (non-APEC) strains. De Carli *et al*. (2015), reported the pathogenicity of the APEC strain in the presence of five VGs.

Recently, the pathogenicity of APEC has been evaluated by the existence of at least three VGs, according to the genetic criteria, which enable them to survive an extra-intestinal life

(Varga *et al.* 2018; Ievy *et al.* 2020). Most recently Tohmaz *et al.* (2022) have evaluated the pathogenicity of APEC by the existence of at least one VG. Despite the genetic diversity of APEC, all the results obtained by different studies demonstrated the ability to differentiate APEC from NON-APEC based on a combination of frequently occurring VGs. Johnson *et al.* (2008b) used a large sample size, combined with extensive genotyping to select VG markers for APEC. Despite the variation in the frequency of these five VGs in different overseas studies, a significant association between them and APEC has been reported (Hussein *et al.*, 2013; Dissanayake *et al.*, 2014; de Oliveira *et al.*, 2015; Tohmaz *et al.* 2022). This study was carried out to investigate avian pathogenic *Escherichia coli* in diseased Chickens from commercial farms in Sokoto and Kebbi States, Northwest Nigeria.

MATERIALS AND METHODS

Study Areas

The study was carried out in two States; Sokoto and Kebbi States

The study was carried out in two States; Sokoto and Kebbi States in Northwest, Nigeria. Sokoto State is geographically located in the North Western part of Nigeria between the longitudes 4°8'E and 6°54'E and latitudes 12°N and 13°58'N, shares borders with Niger republic to the north, Kebbi State to the south and Zamfara State to the east (NPC, 2006). (NPC, 2006).

While Kebbi State is with a total population of 3,238,628 as per the 2006 National census comprising 21 Local Government Areas, four (4) It lies between latitudes 10° 051 and 13° 271N of the equator and between longitudes 3° 351 and 6° 031W of Greenwich. The state shares an international border with the Republic of Benin and the Republic of Niger to the West and North respectively. It also shares domestic borders with Sokoto, Zamfara, and Niger States. (FDLPS, 2011).

Sample Size Estimation

The minimum sample size for this study was determined by the formula

$$n = t^2 x\ p^{exp} (1-p^{exp}) / d^2$$ (Thrusfield, 2005)

Where n=sample size, t^2=the score for a giving interval which is 1.96(S.E) at 95%, confidence interval, p^{exp}=Known or estimated prevalence, and d^2=precision at 0.05.

The sample was calculated at 8.0% prevalence, (Ejeh *et al.*, 2017), at 95% confidence interval, with desired precision of 5%.

$$n = (1.96)^2 \times 0.08 \times (1-0.08) / (0.05)^2,$$

$$n = 0.307328 \times (0.92) / 0.0025 \quad = \quad 113.096704$$

Thus, n = 113

Two hundred and twenty-five (225) samples were collected to increase the chances of detection. Therefore, a total of 450 samples were collected in the two states, 225 samples from each State.

Ethical Approval to Conduct Study

Ethical approval for sampling and farm data collection was obtained from Sokoto and Kebbi States Ministry of Animal Health, Husbandry and Fisheries Developments dated on 19th September, 2023 and 21st September, 2023 from Sokoto and Kebbi States respectively.

Research Design

The study employed a cross-sectional approach to obtain samples from diseased Chickens in commercial farms from Sokoto and Kebbi States. Two hundred and twenty-five (225) samples were collected from each State, making a total of four hundred and fifty samples (450) that were collected in this study to have geographical spread, 75 samples each were collected from the three senatorial zones in each State.

Sampling Method

Sampling was conducted in Sokoto and Kebbi States. Purposive sampling (based on the presenting clinical signs of diarrhea) as described by Valerie and John (1997) was used for sampling Chickens farms. Upon visitation of the farms simple random sampling was used to select among the diseased Chickens in a pen and cloacal swabs were collected from diseased Chickens.

Sample Collection

The sampling frame consists of registered commercial poultry farms in the study area. Upon visitation to a poultry farm, written consent was obtained from the farm owner. Twenty-five (25) samples were collected per flock in a farm. With the aid of a gloved hand, a fresh faecal sample was obtained from the cloacal of a randomly selected chickens. A data collection sheet was used to obtain information about the age, chickens type, antibiotic usage, production type, and size of the flock during this visitation (appendix 1). Swabs were transferred into an adequately labeled sample bottle that was transported for processing on ice packs to Fleming's laboratory of the Veterinary Teaching Hospital of Usmanu Danfodiyo University, Sokoto, Nigeria for culture and isolation. On arriving at the laboratory 2mls of peptone water was added to each sampling bottle and incubated at 37^{o}C overnight for 12 to 24 hours for proliferation of the bacteria.

Bacterial Culture and Isolation

In the laboratory, isolation of *E. coli* was carried out aseptically by streaking each sample from peptone water directly on the MacConkey agar (Oxoid) and incubated aerobically at 37°C for 24hrs, Presumptive *E. coli* colonies, usually pink to red were picked and further streaked on the eosin methylene blue (EMB) agar (Oxoid) and incubated overnight at 37 °C. Colonies with the green metallic sheen on EMB agar were presumed *E. coli* strains. Presumptive isolates of *E. coli* were further subjected to standard biochemical tests (indole, methyl red, Voges-Proskauer, citrate, catalase, and oxidase test) according to ISO 6579 and to a modified protocol described by (Nguyen *et al.*, 2011; García-Meniño *et al.*, 2018; Al Radaideh *et al.*, 2019).

Biochemical Characterization of Isolates

Isolates of *Escherichia coli* were subjected to biochemical tests according to García-Meniño *et al* (2018), Nguyen *et al.* (2019), and Al Radaideh *et al.* (2019). Including the Indole reaction, Methyl red test, Voges Proskauer test, and Citrate utilization test (IMViC), Catalase test, Oxidase test, Triple sugar iron, and Christener's urea agar test.

DNA Extraction by Boiling Method

Genomic DNA extraction was accomplished as described by Blanco *et al*. (2004). Bacterial isolates were first sub-cultured overnight at 37°C in nutrient agar before being suspended in 140µL of sterile nuclease-free water. Bacteria were boiled at 95°C for 15 minutes using a dry bath to disrupt the cells to release the DNA, followed by centrifugation at 14,000 rpm for five minutes. One hundred and twenty microliters (120 µl) of the supernatant was extracted into a sterile labeled Eppendorf tube (DNA template) for PCR. The supernatant containing DNA was then stored at -20°C until used for polymerase chain reaction (PCR).

Molecular Confirmation of *Escherichia coli* Isolates

Confirmation of *E. coli* was done by PCR amplification of the genus specific primers β-d-glucuronidase (*uidA*) gene Table 1 (McDaniels *et al.*, 1998; Gómez-Duarte *et al*., 2010).

Polymerase Chain Reaction protocols and Conditions for Confirmation of *E. coli*

Polymerase chain reaction samples were prepared in a total volume of 20µl in a PCR tube containing 2µl of DNA extract, 0.5µl of each primer (F and R), 7µl of nuclease-free water and 10µl of master mix. The PCR conditions was at 94°C for 5 min (initial denaturation) then the reaction was subjected to 35 cycles of amplification, denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, extension at 72°C for 30 sec; and a final extension at 72°C for 5 min. Positive controls with genomic DNA from *E. coli* ATCC 25922 was included in each PCR run along with a non-template-containing reaction to detect false-positive results. The amplified products were separated by electrophoresis through 1.5% agarose (wt/vol), was stained with 0.5 µg/mL ethidium bromide, and visualized using a GelDoc 1000 fluorescent imaging system (Bio-Rad, USA).

Molecular Differentiation of *E. coli* Pathotypes and Virulence Detection

Following confirmation of *E. coli* by PCR, isolates were screened to differentiate *E. coli* pathotypes ETEC, EPEC and APEC by PCR using specific primer in Table 1, based on specific genes encoding toxins (*iss, stx_1, stx_2, hylA, ompT, iutA, IroN, hlyA* and intimin *eae*) (Vu Khac *et al*., 2006; García-Meniño *et al*., 2018; Awad *et al*., 2020). The bacteria *E coli* isolates that were *eae*-positive and *stx*-negative are identified as EPEC. Isolates that were *lt*- or *st* positive, or both or hylA positive are identified as ETEC. Isolates that were positive to one or more or all of the following virulence genes (*hlyF, iss, iroN, iutA and ompT*) are APEC (Johnson *et al*., 2008b). Positive controls with genomic DNA from *E. coli* ATCC 25922 was included in each PCR run along with a nontemplate-containing reaction to detect false-positive results. The amplified products were separated by electrophoresis through 1.5% agarose (wt/vol), was stained with 0.5 µg/mL ethidium bromide, and visualized using a GelDoc 1000 fluorescent imaging system (Bio-Rad, USA).

Data Analysis

Information from the data sheet was entered into Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) as a database. Chi square was used to check for association between variables and prevalence of *E.coli* infection using SPSS version 26 (IBM, USA). To assess for risk factors, data was exported to Statistical software R for analysis using the glm function. Potential risk factors were selected for univariate regression analysis between specific variables and outcome of *E. coli* status in a farm.

Table 1: Primers for confirmation and pathotyping of *E.coli* strains

Target	Sequence (5' ->3')	Amplicon Size	Reference
uidA-F	GCGTCTGTTGACTGGCAGGTGGTGG	503	Montso *et al.*, (2019)
uidA-R	GTTGCCCGCTTCGAAACCAATGCCT		
*stx*1-F	ATAAATCGCCATTCGTTGACTAC	614	Gannon *et al.*, (1992)
*stx*1-R	AGAACGCCCACTGAGATCATC		
*stx*2-F	GGCACTGTCTGAAACTGCTCC	477	Paton and Paton, (1998)
*stx*2-R	TCGCCAGTTATCTGACATTCTG		

Target	Sequence (5' ->3')	Amplicon Size	Reference
eae-F	ATCTTCTGCGTACTGCGTTCA	384	Yang *et al*., (2020) Paton and Paton, (1998)
eae-R	CATTATGGAACGGCAGAGGT		
hyla-F	AGCTGCAAGTGCGGGTCTG	534	Paton and Paton, (1998)
hyla-R	TACGGGTTATGCCTGCAAGTTCAC		
hlyF-F	TCGTTTAGGGTGCTTACCTTCAAC	450	Johnson *et tal*., (2008)
hlyF-R	TTTGGCGGTTTAGGCATTCC		
IutA-F	GGCTGGACATCATGGGAACTGG	302	
IutA-R	CGTCGGGAACGGGTAGAATCG		
IroN-F	AAGTCAAAGCAGGGGTTGCCCG	553	
IroN-R	GACGCCGACATTAAGACGCAG		
Iss-F	CAGCAACCCGAACCACTTGATG	323	
Iss-R	AGCATTGCCAGAGCGGCAGAA		
OmpT-F	ATCTAGCCGAAGAAGGAGGC	496	
OmpT-R	CCCGGGTCATAGTGTTCATC		

RESULTS

A total of 450 cloacal swab samples were collected in Sokoto and Kebbi States from diseased Chickens in commercial farms. Two hundred and twenty-five samples (225) were collected from each State. Following culture and isolations, one hundred and ninety (84.4%) and one hundred and eighty (80%) samples were positive for *E. coli* from Sokoto and Kebbi States respectively. Biochemical test results revealed the sum of one hundred and four samples (23%) were positive for *E. coli,* fifty-one (22.7%) and fifty-three (23.7%) samples from Sokoto and Kebbi States respectively. Molecular detection using the *uidA* gene confirmed (Figure 1) a total of one hundred and one (22.4%) samples were *E. coli* infections from diseased Chickens in commercial farms from Sokoto and Kebbi States, fifty-one (22.7%) samples from Sokoto State and fifty (22.2%) samples from Kebbi State as shown in Table 2.

The five genes *iroN*, *ompT*, *hlyF*, *iss,* and *iutA* are considered minimal predictors of APEC virulence-associated genes (VAG) (Johnson *et al*, 2008; Tohmaz *et al* 2022). Polymerase chain reaction (PCR) amplification of these genes using simplex and multiplex PCR is shown in Figures 2 - 4. In the current study, the virulotyping of the isolates were carried out which resulted in eleven patterns. Isolates were also scored based on the number of VAG possessed. The virulence scores (VS) of the strains were in the range of 0 to 5. The most prevalent VS was 5 (67/101; 66.34%) table 3. Moreover, the most prevalent VAGs were *ompT* (99/101; 98.02%) and *iutA* (99/101; 98.02%) Figure 5. The prevalence of the genes *hlyF, iss,* and *iroN* were defined as 97 (96.04%), 95(94.06%), and 74 (74.26%), respectively as shown in Figure 5.

Table .2: Identification Workflow of *E.coli* Isolates from Diseased Chickens in Commercial Farms from Sokoto and Kebbi States

Methods	No of samples			No of positive			Percentage (%)		
	Sokoto	**Kebbi**	**Total**	**Sokoto**	**Kebbi**	**Total**	**Sokoto**	**Kebbi**	**Total**
Culture	225	225	450	190	180	370	84.4	80.0	164.4
Biochemical	190	180	370	51	53	101	22.7	23.7	46.4
PCR	51	53		51	50		22.7	22.2	22.5

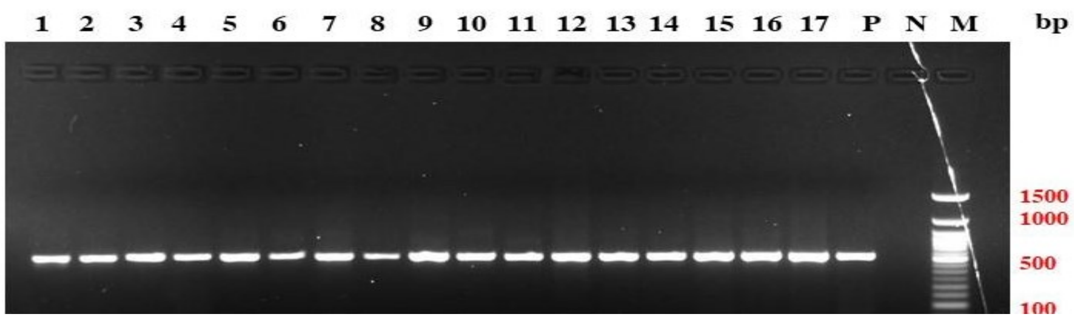

Figure 1: PCR Detection of *uidA* gene in *E. coli* Isolates in Diseased Chickens from Sokoto and Kebbi States. The product Yielded 503 bp typical of *E.coli*. Lane 1-17: Positive isolates, P: Positive control (*E. coli* ATCC 25922), N: Negative control (nuclease-free water), M: 100 bp DNA ladder (Trans-gene Biotech, China)

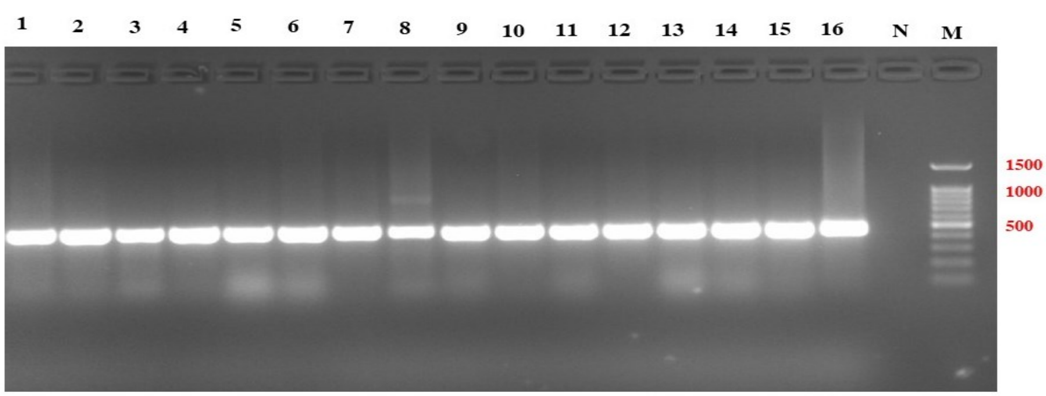

Figure 2: PCR Detection of *hlyF* Gene in *E.coli* Isolates in Diseased Chickens from Sokoto and Kebbi States. The Product Yielded 450 bp Typical of the *hlyF* gene. Lane 1 to 16 are positive isolates having the gene. N: Negative control (nuclease-free water), M: 100 bp DNA ladder (Trans-gene Biotech, China)

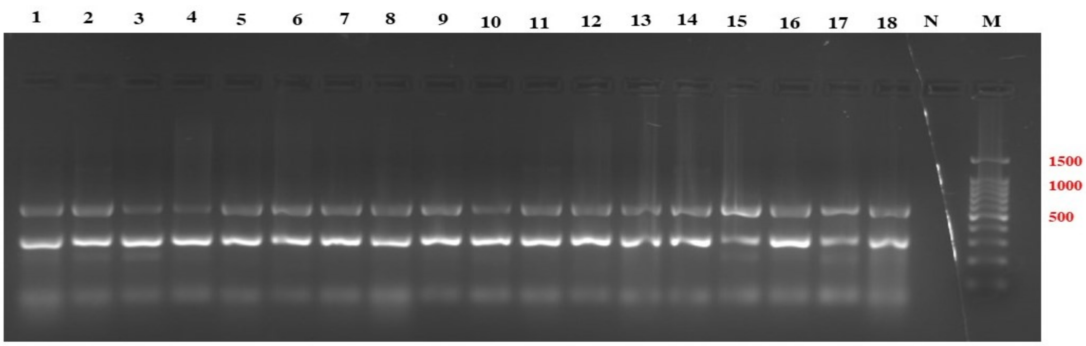

Figure 3: Multiplex PCR Detection of *OmpT* and *IutA* Genes in *E.coli* Isolates in Diseased Chickens from Sokoto and Kebbi States. The Product Yielded 496 and 302 bp Typical of *OmpT* and *IutA* Genes respectively. Lane 1-18: Positive isolates, N: Negative control (nuclease-free water), M: 100 bp DNA ladder (Trans-gene Biotech, China)

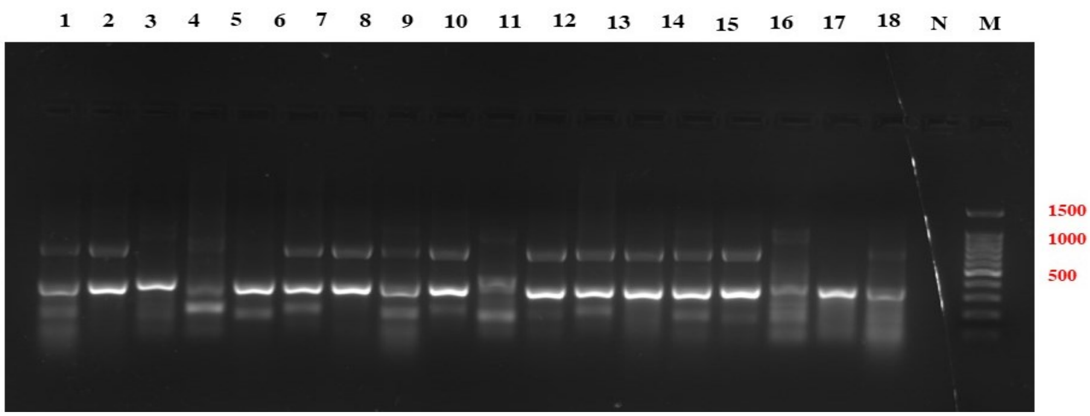

Figure 4: Multiplex PCR Detection of *Iss* and *IroN* Genes in *E.coli* Isolates in Diseased Chickens from Sokoto and Kebbi States. The Product Yielded 323 and 553 bp Typical of *Iss* and *IroN* Genes respectively. Lane 1-18: Positive Isolates for *Iss,* Lane 1 – 2, 7 – 10, 12 -15 and 18 are positives isolates for *IroN* while Lane 3 – 5, 11, 16, and 17 are negatives Isolates for *iroN.* N: Negative control (nuclease-free water), M: 100 bp DNA ladder (Trans-gene Biotech, China)

Table 3: Avian Pathogenic *Escherichia coli* (APEC) Virulence-Associated Genes (VAG) and Virulence Score (VS)

Virulence Associated Genes (VAGs)	Virulence Score (VS)	*E. coli* Strains	Total *E. coli* Strains	Percentage (%) *E. coli* Strains
One VAGs	0	0	0	0
	1	0	0	0
Two VAGs	2	0	0	0

Virulence Associated Genes (VAGs)	Virulence Score (VS)	*E. coli* Strains	Total *E. coli* Strains	Percentage (%) *E. coli* Strains
hlyF, iss, iutA	3	1		
Iss, iutA, ompT	3	1		
hlyF, ompT, iutA	3	2	4	3.96
hlyF, ompT, iss, iutA	4	21		
hlyF, iss, iroN, iutA	4	1		
hlyF, iroN, ompT, iutA	4	4		
hlyF, iss, iroN, ompT,	4	1		
iss, iroN, ompT, iutA	4	3	30	29.70
hlyF, iss, iroN, ompT, iutA	5	67	67	66.34
Total		**101**	**101**	**100**

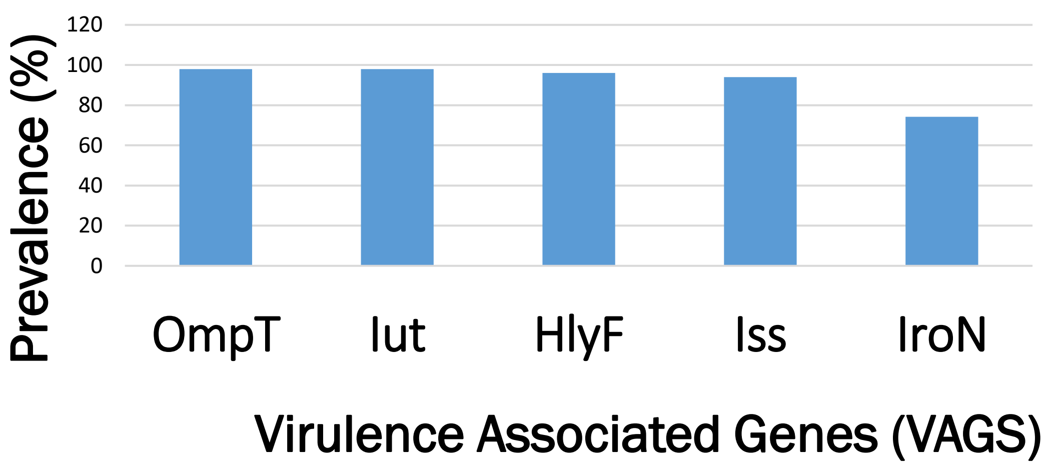

Figure 5: Prevalence of Virulence Associated Genes (VAGS) of APEC

DISCUSSIONS

From the study a total of 450 cloacal swabs were collected, screened and analyzed using standard culture, isolation biochemical characterization, and molecular techniques. Three hundred and seventy 370 (82.2%) were positive to culture and isolation while one hundred and four 104 (23.1%) were positive to biochemical tests. Following polymerase chain reaction techniques only one hundred and one 101 (22.4%) isolates were confirmed *Escherichia coli*.

Pathotyping of all the confirmed *Escherichia coli* isolates were carried out to determine the virulence types. The study conducted by Johnson *et al.* (2008b) aimed to identify a minimum number of virulence-associated genes (VGs) that could identify APEC. In their study, 124 *E. coli* isolates of known pathogenicity underwent extensive genotyping and were screened for the presence of 46 virulence genes VGs. The author identified five VGs (*iutA*, *iss*, *ompT*, *iroN,* and *hlyF*) that were significantly associated with APEC strains. Johnson *et al*. (2008b) developed a pentaplex-PCR, targeting these five VGs, and subsequently screened 994 *E. coli* (794 APEC and 200 AFEC) to validate the selection of the five VGs. The results showed that highly pathogenic APEC strains harboured 5 VGs while 4 and 3 VGs were found among medium and low pathogenic APEC strains, respectively. The VGs in non-APEC isolates were 1-2. In this current study, APEC virulence-associated genes and virulence score were determined using five genes *iroN*, *ompT*, *hlyF*, *iss,* and *iutA* which are considered the minimal predictors of APEC virulence-associated genes (VAG) (Johnson *et al*., 2008b; Tohmaz *et al*., 2022). Amplification of these virulence-associated genes was carried out by simplex and multiplex PCR methods in the present study and the virulotyping of the isolates were carried out which resulted in nine (9) different patterns. Isolates were also scored based on the number of VAG possessed. The strains' virulence scores (VS) ranged from zero to five (0 to 5). The most prevalent VS was 5 (67/101; 66.34%). Moreover, the most pervasive VAGs were *ompT* (99/101; 98.02%) and *iutA* (99/101; 98.02%). The prevalence of the genes *hlyF, iss,* and *iroN* was 96.04%, 94.06% and 74.26%, respectively.

In the present study, the most prevalent VS was 5 (67/101; 66.34%), which concurred with the most pervasive VS 5 (48/72; 66.66%) reported by Tohmaz *et al*. (2022). The most pervasive VAGs were *ompT* (99/101; 98.02%) and *iutA* (99/101; 98.02%). The finding of *ompT* (99/101; 98.02%) as one of the two most VAGs agrees with the study findings of Tohmaz *et al*. (2022) who revealed *ompT* (68/72; 94.4%) as one of the two most VAGs in APEC but disagreed with *hlyF* (68/72; 94.4%) as one of two most VAGs in his results as revealed in this study findings *iutA* (99/101; 98.02%) is one of the two most VAGs in APEC.

Based on the genetic criteria for the pathogenicity, isolates containing at least three virulence genes were considered the APEC strains, and isolates containing less than three virulence genes were considered the Avian nonpathogenic *Escherichia coli* (non-APEC) strains. The pathogenicity of APEC strains is multifactorial, based on the presence and expression of different groups of VGs (Won *et al*., 2009). De Carli *et al*. (2015), reported the pathogenicity of the APEC strain in the presence of five VGs. Recently, the pathogenicity of APEC has been evaluated by the existence of at least three VGs, according to the genetic criteria, which enable them to survive an extra-intestinal life (Varga *et al*., 2018; Ievy *et al*., 2020). Most recently Tohmaz *et al*., (2022) have evaluated the pathogenicity of APEC by the existence of at least one VG. The present study revealed that, Out of 101 (100%) confirmed *Escherichia coli* isolates, all isolates were found to be APEC. Among 101 confirmed *Escherichia coli* isolates, none of the strains contained one virulence gene, no strains contained only two virulence genes, four (4) strains contained

three virulence genes, and 30 strains contained four virulence genes. All the five virulence genes *iss, ompT, hlyF*, and *iroN* were detected in 67 strains of the confirmed *Escherichia coli* isolates. Therefore, all the confirmed 101 (100%) *Escherichia coli* isolates have a minimum of either three (3), four (4), or five (5) VGs (*iutA*, *iss*, *ompT*, *iroN* and *hlyF*) which are the minimal predictors of APEC, are therefore confirmed to be all APEC pathotypes.
Globally, colibacillosis is considered one of the major problems that affects the poultry industry and translates into multimillion-dollar losses annually (Johnson *et al*., 2008b; Nolan *et al*., 2013a; Guabiraba & Schouler, 2015). Interestingly, although there has been some development of vaccines to alleviate this problem, none have been effective in fully controlling this infection or disease (Lynne *et al*., 2007; Lynne *et al*., 2012). Despite the intensive research globally, the genetic diversity of APEC makes it hard to reach a consensus on definition of APEC (Rodriguez-Siek *et al*., 2005a; Lynne *et al*., 2007; Johnson *et al*., 2008b).

Conclusions

The present study revealed that, Out of 101 (100%) confirmed *Escherichia coli* isolates, all isolates were found to be APEC. Among 101 confirmed *Escherichia coli* isolates, none of the strains contained one virulence gene, no strains contained only two virulence genes, four (4) strains contained three virulence genes, and 30 strains contained four virulence genes. All the five virulence genes *iss, ompT, hlyF*, and *iroN* were detected in 67 strains of the confirmed *Escherichia coli* isolates. Therefore, all the confirmed 101 (100%) *Escherichia coli* isolates have a minimum of either three (3), four (4), or five (5) VGs (*iutA*, *iss*, *ompT*, *iroN* and *hlyF*) which are the minimal predictors of APEC, are therefore confirmed to be all APEC pathotypes.

Recommendations

Based on the study findings, avian pathogenic *Escherichia coli* infection in diarrhea chickens from commercial farms in the study areas has been established. The following suggestions are therefore recommended:

- Farms, especially large-scale commercial ones, should implement stricter biosecurity practices to reduce the spread of *E. coli*, particularly in deep litter systems.
- Regular disinfection and restriction of farm access should be prioritized.
- Given the lower prevalence of *E. coli* infection in battery cage systems, commercial farms could consider transitioning from deep litter to cage systems or hybrid systems to reduce infection rates.

- Introducing vaccines specifically designed to target avian pathogenic *E. coli* (APEC) strains could significantly lower infection rates.

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