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Identification of Antibiotic Resistance Pattern, and Detection of Virulence Genes *iss,* and *ompT* in Avian Pathogenic *Escherichia coli* from Broiler Chickens in Chitwan, Nepal

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Authors' contributions

This work was carried out in collaboration among all authors. Author SS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors RKB, HBB, NRM, SS, HM and MN managed the analyses of the study. Authors SS, SS and NRM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study aimed to identify, evaluate the antibiotic resistance pattern and detect virulence genes *iss,* and *ompT* in avian pathogenic *Escherichia coli* (APEC) from broiler chickens in central Nepal. To determine the antibiotic resistance pattern of the obtained isolates, the Kirby-Bauer disc

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diffusion method was used with six different commercial antibiotic discs: Amikacin, Gentamycin, Ciprofloxacin, Doxycycline, Chloramphenicol and Levofloxacin. A polymerase chain reaction (PCR) assay was used for the selected isolates (n=40) to screen the presence of the *iss* and *ompT* genes after the extraction of DNA using the boiling method Out of 60 suspected Colibacillosis liver samples, 40 were confirmed as *E. coli* positive The antibiogram profile revealed maximum resistance to Doxycycline (87.5%), followed by Levofloxacin (72.5%), Ciprofloxacin (67.5%), Chloramphenicol (40.0%), Gentamycin (32.5%) and Amikacin (10.0%).. The presence of the *iss* and *ompT* genes was found to be 100.0% and 90.0%, respectively. APEC was found to be highly resistant to most of the antibiotics. Virulence-associated genes *iss* and *ompT* were obtained at high percentages from Colibacillosis suspected broiler chickens in Chitwan, Nepal. These finding suggests that the judicial use of antimicrobials is compulsory to check antibiotic resistance and Colibacillosis outbreaks in poultry farms.

Keywords: Antibiotic resistance; virulence genes; iss; ompT; avian pathogenic Escherichia coli; broiler; PCR.

ABBREVIATIONS

AFU : Agriculture and Forestry University
AMK : Amikacin
APEC : Avian Pathogenic E. coli
ATCC : American Type Culture Collection
BHIB : Brain Heart Infusion Broth
bp : base pair
CIP : Ciprofloxacin
CHL : Chloramphenicol
CL : Colistin
CLSI : Clinical and Laboratory Standards Institute
DNA : Deoxyribose Nucleic Acid
DOXY : Doxycycline
DW : Distilled water
EMB : Eosin Methylene Blue
ExPEC : Extraintestinal Pathogenic E. coli
FAO : Food and Agriculture Organization
GEN : Gentamicin
KOH : Potassium Hydroxide
LB : Luria Bertani
LEV : Levofloxacin
MAC : MacConkey Agar
MAR : Multiple Antibiotic Resistances
MDR : Multiple Drug Resistance
MHA : Muller Hinton Agar
MIO : Motility Indole Ornithine
MR : Methyl Red
NADIL : National Avian Disease Investigation Laboratory
NMEC : Neonatal meningitis causing Escherichia coli
PCR : Polymerase chain reaction
rpm : Rotation Per Minute
SXT : Sulfamethoxazole
TBE : Tris-bore EDTA buffer
TSI : Triple Sugar Iron
UPEC : Urinary Pathogenic E. coli
UV : Ultra-violet

1. INTRODUCTION

Escherichia coli (*E. coli*), mostly colonized in the gastrointestinal tract of warm blooded animals and birds, is a gram-negative, rod shaped, flagellated, non-sporulating, and facultative anaerobic bacteria belonging to the Enterobacteriaceae family of class Gammaprotobacteria [1,2]. Most *E. coli* strains are nonpathogenic; however, some are pathogenic. Some pathogenic strains of *E. coli* invade various organs of birds, which leads to pericarditis, air sacculitis, perihepatitis, peritonitis, and other extraintestinal diseases, collectively termed Colibacillosis [3,4]. Avian pathogenic *E. coli* (APEC) strains that fall under the category of extraintestinal pathogenic *E. coli* (ExPEC) are highly associated with diverse diseases and are responsible for great economic losses in the avian industry [5].

Antibiotic therapy is one of the prime measures for controlling the morbidity as well as mortality caused by APEC infection. Many studies have suggested the use of antibiotics in chicken broilers as a disease preventive measure and a growth promoter [6,7]. Since the haphazard use of antibiotics leads to the occurrence and development of resistant isolates, they need to be used prudently to restore their therapeutic usefulness in both humans and animals [8]. European countries along with others have reported on the widespread use of large amounts of antibiotics without any professional consultation or supervision [7,9]. The utilization of antibiotics in food-producing animals is associated with several adverse effects, such as modification of the intestinal flora, appearance of antibiotic residues in food particles, impacts on the public environment, and emergence of antibiotic resistance in microorganisms [10,11]. Such microbes have challenged the cure of zoonotic diseases, and their transmission from animals to humans has threatened the public health care sectors [12].

The pathogenic ability of the APEC strain is determined by a broad range of virulence factors that are coded by virulence-associated genes such as *iutA, papC, iroN, iss, ompT, cva/cvi, tsh, hylF, astA, iucD, and irp-2.* Of the eleven genes reported worldwide, *iss* and *ompT* genes are considered the most widespread virulence genes in APEC strains [13]. The increased serum survival *(iss*) gene plays a major role in resistance against serum complement. *E. coli* resists complement by producing Iss proteins. This protein inhibits classical and alternate pathways of the complement system. It causes a 20-fold increase in complement resistance and a 60-fold increase in virulence towards poultry [14]. As the *iss* gene has been found more frequently among pathogenic than nonpathogenic strains, a strong association is suggested between the *iss* gene and pathogenicity of APEC of various serogroups but not with fecal *E. coli* isolates from apparently healthy birds [15]. Another virulence gene called the *ompT* gene encodes the OmpT protein, which is an aspartyl protease found on the outer membrane of *E. coli*. This *ompT* gene is also found on some gram-negative species of bacteria that hydrolyse peptide materials that are harmful to the bacterium and hence increase the virulence of that gene [16].

In Nepal, poultry farming has been growing tremendously over a few decades. It shares a total of 8% of AGDP and 4% of GDP [17]. The Chitwan district of Nepal is the major broiler producing district containing more than 1,365 broiler farms [18]. Studies on the molecular characterization of virulence-associated genes, such as *iss* and *ompT,* responsible for Colibacillosis are scarce in Nepal. Most of the studies have only concentrated on prevalence studies. PCR, a confirmatory test, highlights the importance of molecular detection of virulence genes and is easier to apply than other methods [13,14,19]. This type of study could provide further evidence and platform for other researchers to conduct research on candidate vaccines that might be proven successful in eliminating Colibacillosis infections in broiler chickens of Nepal [19]. Hence, the study aimed to identify, evaluate the antibiotic resistance pattern, and detect virulence genes *iss* and *ompT* in APEC from broiler chickens in central Nepal.

2. METHODS

2.1 Study Duration and Site

This study was conducted from September 2018 to February 2019 in Chitwan District, which is located in the southwest part of Baghmati Province. The samples were obtained from the National Avian Disease Investigation Laboratory (NADIL), Bharatpur, Chitwan, where dead birds from different areas of Chitwan are taken for postmortem and disease diagnosis. Identification, antibiotic resistance patterns, and gene detection were performed at the Veterinary Microbiology Lab, Faculty of Animal Science,

Veterinary Science and Fisheries (FAVF), Agriculture and Forestry University (AFU), Chitwan.

2.2 Sample Collection

Purposive sampling was performed. A total of 60 liver samples of broilers from NADIL were collected aseptically in a sterile vial containing 1 ml of sterile peptone water. It was then immediately transported on a cold chain box with ice packs to the microbiology laboratory of FAVF, Chitwan. All liver samples were taken from the broilers that were non-vaccinated, between 4-8 weeks of age and with no history of antibiotic therapy after one week of age.

2.3 Identification of *E. coli*

Streaking was performed directly on MacConkey agar (MAC) plates and left for incubation at 37°C aerobically for 24 hours. Afterwards, a single isolated colony from MAC agar was subcultured onto the EMB agar plate and incubated at 37°C aerobically for 24 hrs. Green metallic sheen with dark centered colonies was observed on EMB agar plates. Various morphological characteristics, such as size, shape, surface texture, edge and elevation, opacity, color, etc. of the suspected colonies on different agar media developed within 18-24 hrs of incubation was carefully studied and recorded. Suspected purified colonies were smeared, fixed and stained with Gram's staining. As *E. coli* is a gram-negative bacterium, the bacterial smear should appear pinkish with the shape of bacilli.

2.4 Identification Tests

Biochemical tests, such as motility, indole, methyl red, Voges- Proskauer, simmon citrate, triple sugar iron (TSI), motility indole ornithinase (MIO) test, catalase, and oxidase test, were performed. The criteria was followed for various biochemical tests to confirm *E.coli*, as depicted in Table 1.

2.5 Antimicrobial Susceptibility Testing

The antibiotic susceptibility test was performed using the modified Kirby-Bauer disk diffusion method as suggested by the Clinical and Laboratory Standards Institute (CLSI) [20]. All 40 APEC isolates were tested for six different antimicrobial agents on Muller Hinton agar (HiMedia). Isolates of *E. coli* were inoculated in brain heart infusion broth (BHIB) and incubated at 37°C for 6 hours. The turbidity of the sample was adjusted to a 0.5 McFarland standard by dilution. Instantly, after dilution, a sterile swab was dipped into the inoculum and streaked over the entire surface of the Muller Hinton agar three times. Six suitable antibiotic discs were selected. Disks of Amikacin (30 mcg), Gentamycin (10 mcg), Ciprofloxacin (5 mcg), Levofloxacin, Chloramphenicol (30 mcg), and Doxycycline (25 mcg) were used.

2.6 DNA Extraction, Quantification and Primers

The rapid boiling method was performed for deoxyribose nucleic acid (DNA) extraction [21]. Quantification and purity detection of DNA extracted from *E. coli* culture was performed spectrophotometrically at 260 nm and 280 nm using a Nanodrop Spectrophotometer (Quawell, UV-Vis Spectrophotometer Q5000). Forward and reverse primers of both the *iss* and *ompT* genes were used, as shown in Table 2 [22]. The polymerase chain reaction (PCR) primers used in the study were 323 bp for the *iss* gene and 496 bp for the *ompT* gene.

Table 1. Criteria for various biochemical tests to confirm *E. coli*

Test or substrate	Result		*E. coli*
	Positive	**Negative**	**Reaction**
Indole test	Red color at surface	Yellow color at surface	+
Voges-proskauer test	Pink-to-red color	No color change	-
Methyl red test	Diffuse red color	Diffuse yellow color	+
Simmon's citrate test	Growth: blue color	No growth: no color change	-
Catalase test	Gel formation	No gel formation	+
Oxidase test	Bubble formation	No bubble formation	-

"+" positive; "-" negative

Table 2. Primer sequences and their details

Genes	Oligonucleotide sequence (5´-3´)	Amplicon size (bp)
Iss	F: CAGCAACCCGAACCACTTGATG R:AGCATTGCCAGAGCGGCAGAA	323 bp
ompT	F:TCATCCCGGAAGCCTCCCTCACTACTAT R:TAGCGTTTGCTGCACTGGCTICTGATAC	496 bp

F = Forward, R= Reverse primers

2.7 Polymerase Chain Reaction (PCR)

The PCR mixture for the *iss* and *ompT* genes was set up in a final volume of 20 µl. It contained nuclease-free water, Hot Start Taq 2X Master Mix, forward primer, reverse primer, and DNA template of volumes 6 µl, 10 µl, 1 µl, 1 µl, and 2 µl, respectively. The Bio-Rad T100™ Thermal Cycler (Bio-Rad, USA) was used for the PCR thermal cycling conditions with a predenaturation step at 94°C for 4 min and a denaturation step at 94°C for 30 secs with annealing temperatures at 60°C for 1 min and an elongation temperature at 68°C for 2 min. The cycle was repeated from step 2 for 35 cycles. After a final extension step at 72°C for 7 min, the sample was stored at 4°C for infinity. Conventional PCR was performed to amplify two virulence-associated *E. coli* genes, i.e. *iss* and *ompT.* The amplified PCR products were resolved by electrophoresis in a 1.5% agarose gel. Staining of the gel was performed using SYBR safe (Invitrogen, USA). The gel electrophoresis was ran and set at 85 V and 90 A for 75 min. The gel was removed from the tray and finally visualized under a UV trans-illuminator (Platinum Q9, Uvitech Cambridge). Positive and negative controls were used for the research. Pure *E. coli* samples preserved in the veterinary microbiology lab of AFU were used as positive controls. Likewise, the Salmonella vaccine was used as a negative control.

2.8 Statistical Analysis

Data entry and analysis were performed using Microsoft Excel 2010.

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3. RESULTS

Out of 60 diseased birds clinically diagnosed with Colibacillosis, 40 isolates were biochemically identified as *E. coli* with a prevalence rate of 66.67%.

Among the 60 liver samples obtained from Broiler chickens, 40 were confirmed to be *E. coli* positive through cultural and biochemical tests, as predicted in Table 3.

The antibiotic profile of 40 APEC isolates showed the highest resistance against Doxycycline (87.5%), followed by Levofloxacin (72.5%), Ciprofloxacin (67.5%), Chloramphenicol (40.0%), Gentamycin (32.5%), and, Amikacin (10.0%). Likewise, Ciprofloxacin (25%) showed the highest intermediate resistance. The highest sensitivity was shown against Amikacin (82.5%). All isolates were resistant against at least one antibiotic, as depicted in Figs. 2 and 3.

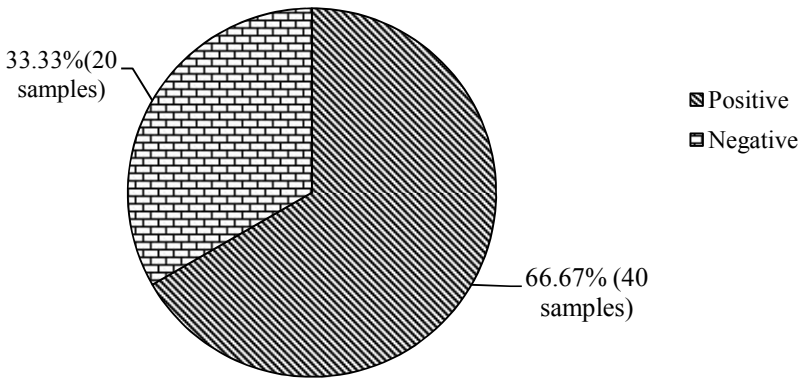

Fig. 1. Prevalence rate of *E. coli* in clinically suspected cases

Table 2. Cultural and biochemical profiles of bacteria obtained from liver samples

Sample no	Growth on MacConkey agar (Pink colonies)	Growth on EMB agar (Green Metallic colonies)	Indole test	Methyl Red test	Voges-Proskauer test	Citrate test	Catalase test	Oxidase test	Diagnosis (Conformation of *E. coli*)
1.	+	+	+	+	-	-	+	-	+
2.	+	+	+	+	-	-	+	-	+
3.	+	+	+	+	-	-	+	-	+
4.	+	+	+	+	-	-	+	-	+
5.	+	+	+	+	-	-	+	-	+
6.	+	+	+	+	-	-	+	-	+
7.	+	+	+	+	-	-	+	-	+
8.	+	+	+	+	-	-	+	-	+
9.	-	-	+	+	-	-	+	-	-
10.	+	+	+	+	-	-	+	-	+
11.	+	+	+	+	+	+	+	-	-
12.	+	+	+	+	+	+	+	-	-
13.	+	+	+	+	-	-	+	-	+
14.	+	+	+	+	-	-	+	-	+
15.	+	+	+	+	-	-	+	-	+
16.	+	+	+	+	-	-	+	-	+
17.	-	-	-	-	-	-	+	-	-
18.	-	-	-	-	-	-	+	-	-
19.	-	-	-	-	-	-	+	-	-
20.	-	-	-	-	-	-	+	-	-
21.	-	-	-	-	-	-	+	-	-
22.	-	-	-	-	-	-	+	-	-
23.	+	+	+	+	-	-	+	-	+
24.	+	+	+	+	-	-	+	-	+
25.	+	+	+	+	-	-	+	-	+
26.	+	+	+	+	-	-	+	-	+
27.	+	+	+	+	-	-	+	-	+
28.	+	+	+	+	-	-	+	-	+
29.	+	+	+	+	-	-	+	-	+
30.	+	+	+	+	-	-	+	-	+
31.	+	+	+	+	-	-	+	-	+
32.	+	+	+	+	-	-	+	-	+

Sample no	Growth on MacConkey agar (Pink colonies)	Growth on EMB agar (Green Metallic colonies)	Indole test	Methyl Red test	Voges-Proskauer test	Citrate test	Catalase test	Oxidase test	Diagnosis (Conformation of *E. coli*)
33.	+	+	+	+	-	-	+	-	+
34.	+	+	-	-	-	-	+	-	-
35.	+	+	-	-	-	-	+	-	-
36.	+	+	-	-	-	-	+	-	-
37.	+	+	-	-	-	-	+	-	-
38.	+	+	-	-	-	-	+	-	-
39.	+	+	-	-	-	-	+	-	-
40.	+	+	-	-	-	-	+	-	-
41.	+	+	-	-	-	-	+	-	-
42.	+	+	+	+	-	-	+	-	+
43.	+	+	+	+	-	-	+	-	+
44.	+	+	+	+	-	-	+	-	+
45.	+	+	+	+	-	-	+	-	+
46.	+	+	+	+	-	-	+	-	+
47.	+	+	+	+	-	-	+	-	+
48.	+	+	+	+	-	-	+	-	+
49.	+	+	+	+	-	-	+	-	+
50.	+	+	+	+	-	-	+	-	+
51.	+	+	+	+	-	-	+	-	+
52.	+	+	+	+	-	-	+	-	+
53.	+	+	+	+	-	-	+	-	+
54.	+	+	+	+	-	-	+	-	+
55.	+	+	+	+	-	-	+	-	+
56.	+	+	+	+	-	-	+	-	+
57.	+	+	+	+	-	-	+	-	+
58.	+	+	-	-	-	-	+	-	-
59.	+	+	-	-	-	-	+	-	-
60.	+	+	-	-	-	-	+	-	-

"+" positive; "-" negative

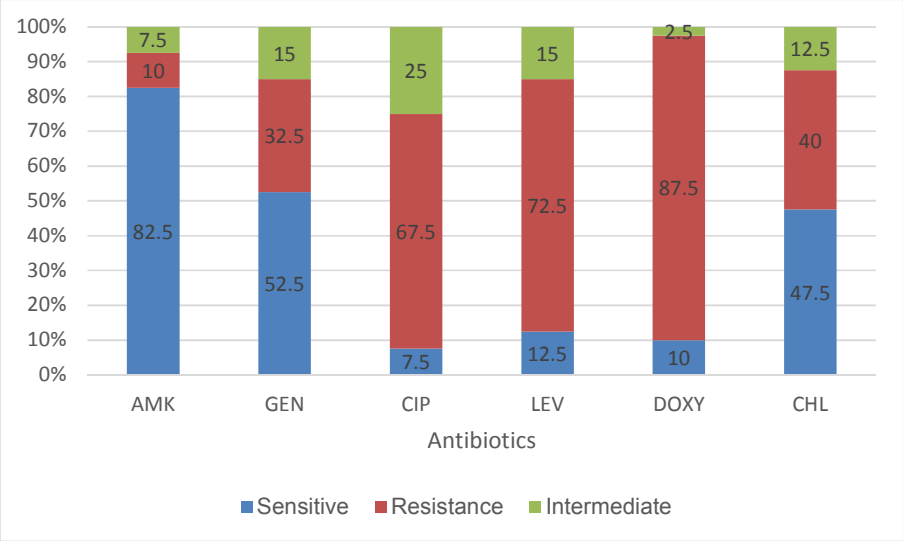

Fig. 2. Antibiotic resistance pattern of APEC isolates of broilers

Abbreviation: AMK: Amikacin; GEN: Gentamycin; CIP: Ciprofloxacin; LEV: Levofloxacin; DOXY: Doxycycline; CHL: Chloramphenicol

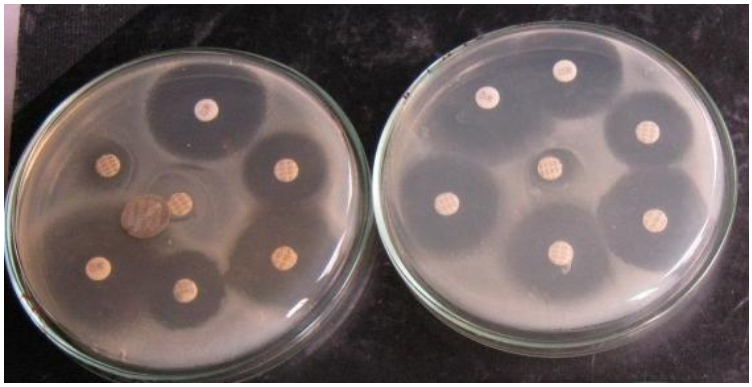

Fig. 3. Antimicrobial susceptibility test of liver samples of broiler chickens

The presence of virulence-associated genes in the *E. coli* isolates was detected using PCR. Out of 40 total confirmed *E. coli* isolates, the *iss* gene was present in all with the highest prevalence of 100.0%, while the *ompT* gene was harbored in 36 isolates, with a prevalence of 90.0%, as illustrated in Figs. 4 and 5.

4. DISCUSSION

Our study identified, evaluated the antibiotic resistance pattern and detected virulence genes *iss* and *ompT* in avian pathogenic *Escherichia coli* from broiler chickens in central Nepal over six months. The study revealed that the 40 isolates were biochemically identified as *E. coli* with a prevalence rate of 66.67%. This was comparatively higher than a study conducted in a similar setting in Egypt, where the prevalence rate was 34.5% [23]. The higher prevalence rate of *E. coli* in our study may be due to differences in the cultural/serological diagnostic tests, lower sample sizes, and persistence of unhygienic farm surroundings, unsanitized water and infections after respiratory diseases in chickens.

Antibiotics are considered an effective measure for the control of Colibacillosis. However, the emergence of multidrug-resistant strains has evolved challenges in the risk reduction of APEC infections. Our study showed a high rate of resistance to the majority of the examined antibiotics. Of the six antibiotics tested, none of

them showed 100% sensitivity against the APEC strain. Gentamycin presented the lowest resistance of 32.5%, and doxycycline showed the highest resistance of 87.5%. This finding is comparable with that of other studies [4,19,24,25]. Alternatively, aminoglycoside amikacin was found to be most effective against 82.5% of the *E. coli* strain, which is slightly lower than that of a study conducted in central Nepal [19]. The antibiotic resistance pattern detected in this current research demonstrates a devastating situation of antibiotic-resistant *E. coli* strains in the Chitwan district of Nepal. Irrational use of antibiotics for nontherapeutic purposes even without consulting professionals may be the reason for the higher drug resistance seen in broiler chickens in Chitwan, Nepal.

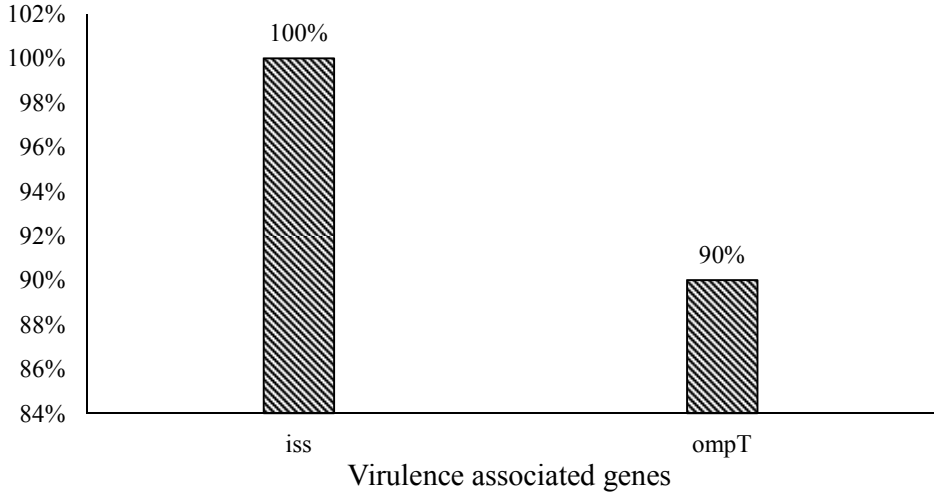

Fig. 4. Detection rates of virulence-associated genes in APEC

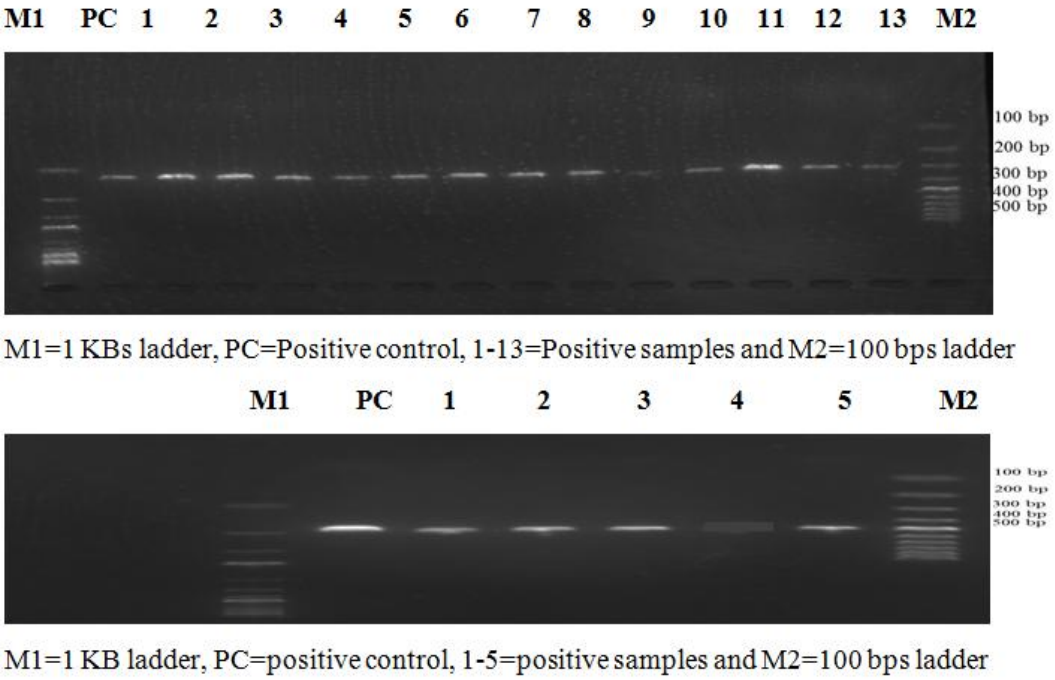

Fig. 5. Detection of the iss and ompT genes, respectively, from positive isolates of E. coli

The frequency of two genes, *iss* and *ompT,* and their role in pathogenicity were analyzed among APEC strains. The *iss* gene was detected in all isolates, showing 100% prevalence, which was consistent with two other previous studies [19,26]. Conversely, the *iss* gene was detected in only 38.5% of the isolates in a study conducted in Egypt [23]. Likewise, our findings showed a 90% detection rate of the *ompT* gene. This is quite similar to the finding reported from Brazil and central Nepal, wherein the detection rate was 100% for the *ompT* gene [3,19]. Conversely, the detection rate of the *ompT* gene was comparatively lower in other studies conducted in China (57.6% and 67%, respectively) [26,27]. The difference in the detection rate of the *iss* and *ompT* genes may be due to differences in the base pair of primers, DNA extraction technique (kit method/direct boiling method), sample difference (liver/trachea/faeces/heart), differences in poultry breeds, geographical differences, environmental factors, etc.

5. CONCLUSION

Our study revealed APEC to be more resistant to most antibiotics. Likewise, virulence-associated genes *iss* and *ompT* were obtained at high percentages from Colibacillosis-suspected broiler chickens. Further study on the pathogenicity of the *iss* and *ompT* genes by taking larger sample sizes may provide ideas for the production of therapeutic vaccines. These findings highlight the essence of the judicial use of antibiotics at an optimum dose to ensure effective treatment of Colibacillosis in broiler chickens. It also urges the need for collaborative efforts from poultry farm owners, veterinarians and government agencies for the prevention and control of avian Colibacillosis in Nepal.

6. LIMITATION AND STRENGTH

There are some limitations to this study. Sample collection was limited to single centre of Chitwan district of Nepal, which along with small sample size shrinks the reliability of the study. Likewise, only two virulence genes were detected in the study. Therefore, the generalizability of the findings remains to be explored. More gene detection from poultry could have strengthen the findings on the status of virulence genes and necessity of novel vaccine production against the Colibacillosis disease in poultry. However, the use of confirmatory biochemical test for *E. coli* detection and molecular technique for gene detection have strengthen this study thus preventing from probable false positive result.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The Research Ethics Committee of Agricultural and Forestry University, Rampur, Chitwan, Nepal granted ethical approval for the study. Animal samples were collected and processed by following animal research ethical guidelines approved by the Ethics Committee of this University.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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