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DETECTION AND ASSOCIATION OF SELECTED VIRULENCE ASSOCIATED GENES AND ANTIBIOTIC RESISTANCE GENES WITH PHYLOGENETIC GROUP IN AVIAN PATHOGENIC *ESCHERICHIA COLI* FROM COMMERCIAL CHICKENS IN NEPAL

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Abstract

The poultry industry faces a significant challenge from avian pathogenic *Escherichia coli* (APEC), posing a threat to food safety chains and resulting in substantial financial losses. We aim to investigate the association of genotypic and phenotypic resistance with the phylogenetic group and virulence genotype in APEC isolated from clinical cases of colibacillosis in commercial broiler, layer, and breeder chickens. We used logistic regression to identify associations among resistance and susceptibility to antibiotic agents, virulence and phylogenetic groups of APEC isolates. The study revealed a significant association between resistance or susceptibility to antibiotic agents (neomycin, ceftriaxone, chloramphenicol, cotrimoxazole and doxycycline) with the phylogenetic groups of APEC isolates, particularly with a high association with phylogenetic group B1. The phylogenetic groups C, D, and E clade were associated with levofloxacin, cotrimoxazole, and doxycycline susceptibility. The most prevalent virulence associated genes (VAGs) (*fimH, iss*^{a}, *traT*^{a}, *sit.chr*., *kpsII*, *iss*^{b}, and *cvi.cva)* in APEC were equally associated with all phylogenetic groups. The carriage of these prevalent genes were significantly associated with phylogenetic group D and E (16 gene out of 57 tested). Additionally, the carriage of *sfa.foc* was significantly associated with B2. *qnrA* genes and *tetB* genes were significantly associated with isolates positive for VAGs. Commercial broiler, layer, and breeder chickens in Nepal harbored APEC with varying susceptibility to the tested antimicrobials, with the majority being resistant to commonly used antibiotics. Most APEC isolates from these chickens may act as reservoirs for antibiotic resistance genes (ARGs), with the colistin resistant gene *mcr1* being the most predominantly detected gene. The work highlights the importance of exploring the interconnections between virulence genotype, genetic background, and antimicrobial resistance (AMR) in APEC strains. It is noteworthy that ARGs and phenotypic resistance alone may not determine AMR due to their inverse relationship, indicating that these chickens could be potential reservoirs for antibiotic resistant APEC. Such insights are crucial for addressing challenges related intriguing VAGs, AMR of poultry production, as well as ensuring food safety and public health.

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Keywords: Antibiotic Resistance Gene, Chicken, Colibacillosis, Phylogenetic Groups, Univariate Analysis, Virulence-Associated Genes.

1. INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) constitutes a subset of *Escherichia coli* (*E. coli)* strains specifically targeting avian hosts, resulting in diverse forms of colibacillosis in poultry [1]. The emergence and persistence of APEC present significant challenges to the poultry industry, leading to economic losses [2] and impacting food safety [3]. The phylogenetic grouping is a comprehensive repository of *E. coli* strains that covers a wide spectrum of genetic diversity of *E. coli* which is invaluable resource for comparative studies and genetic research. APEC serves as a critical model for studying virulence associated genes (VAGs) and antimicrobial resistance (AMR), while the phylogenetic grouping provides a standardized, diverse genetic repository that supports comprehensive phylogenetic and functional studies. Understanding the association between AMR, phylogenetic grouping, and VAGs is crucial for devising effective control strategies. AMR is a major concern associated with APEC, given the escalating resistance to commonly used antimicrobial agents in poultry farming. APEC poses a complex challenge encompassing AMR and VAGs. Therefore, multidisciplinary research efforts are needed to unravel the molecular foundation of APEC pathogenicity to pave the way for infection prevention management and control.

The increasing prevalence of multi-drug resistant (MDR)-APEC (MDR-APEC) is a critical concern that undermines the effectiveness of therapeutic interventions in poultry farming. APEC strains exhibit a concerning tendency to develop resistance to commonly used antimicrobial agents, raising questions about the underlying mechanisms and potential transfer of resistance genes. Both APEC and extraintestinal pathogenic *E. coli* (ExPEC), with potential implications for human disease, share similar serotypes, phylogenetic groups, virulence factors, and disease-causing potential [4, 5]. Contamination of retail poultry meat with APEC intended for human consumption emphasizes the potential zoonotic hazard. Notably, various VAGs associated with pathogenicity islands (PAIs) on large transmissible plasmids are known to enhance APEC virulence. Additionally, several VAGs play a pivotal role in the pathogenesis of APEC infections and usually no single VAG alone is attributable to disease in chickens [6]. These genes encode various factors such as adhesins, invasins, protectins, iron acquisition and toxins that enhance the ability of APEC to adhere to colonize and invade host tissues, evade immune responses, and establish systemic infections [7]. The ability of APEC to cause disease is largely dependent on its VAGs. They give the bacteria the ability to attach themselves to and infiltrate the tissues of their hosts, obtain vital nutrients, generate toxins, elude the host's defense mechanism, and create protective biofilms. Knowing these genes and how they function can help create targeted therapies to manage infections and provide insights into the pathogenic methods used by APEC. VAGs association with phylogenetic group and AMR is critical. Under the selection pressure of antibiotics, their association with AMR enhances the survival and adaptability of APECs. VAGs and phylogenetic marker are

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crucial for classifying and understanding the APECs' evolutionary relationship as well. The challenge lies in identifying specific ARGs, VAGs, their distribution of each among different population, and their role in the progression of APEC infections. Furthermore, the interplay between VAGs and host factors adds another layer of complexity to the pathogenicity of APEC, necessitating targeted research to elucidate these intricate interactions [8]. Phylogenetic analysis is a tool to help classify *E. coli* into types to help infer potential relationship between strains. Classifying APEC strains into phylogenetic groups provides insights into their evolutionary history and adaptation to avian hosts, emphasizing the importance of phylogenetic studies in understanding their evolutionary dynamics.

The rising AMR in APEC strains contributes to their significance, raising global concerns about AMR in bacteria affecting both humans and animals. Reports of MDR-APECs in poultry underscore the collective impact of antibiotic use in the industry. In the Nepalese chicken industry, antibiotics are still being used to treat, prevent, and control bacterial infectious illnesses and stimulate growth.

Several studies in the past have investigated the association between resistance to individual or multiple antimicrobials and the presence of several virulence genes, such as *cvaC*, *iss*, and *iutA*), suggesting that ARGs and virulence genes may be located on the same plasmids [9-11]. The ability of APEC strains to acquire and spread mobile genetic elements (MGEs) that encode VAGs and resistance genes poses challenges not only for poultry health but also for public health, amplifying the risk of transmitting resistance to human pathogens through the food chain by horizontal gene transfer (HGT)[12-19]. However, the specific factors driving the association between phylogenetic grouping, pathogenicity and AMR in APEC remain challenging. Understanding why certain phylogenetic groups are more prone to acquiring and expressing resistance and virulence traits is crucial for predicting the emergence of highly pathogenic MDR-APEC strains. This knowledge is crucial for developing effective strategies to mitigate the impact of APEC on poultry health and to prevent potential spillover of resistance genes into the broader microbial community. Addressing this challenge requires exploring the genetic and environmental factors shaping the phylogenetic diversity of APEC and its implications for disease outcomes.

On-farm studies in Nepal on APEC in commercial chickens are very scarce in Nepal. This study aims to provide a comprehensive understanding of APEC strains, considering various genotypic and phenotypic features and using statistical analysis to identify their potential associations. The goal of this study is to identify and contrast the genetic backbone (phylogenetic group) and virulence genotype with AMR genotype and phenotype at the isolate level, focusing on colibacillosis confirmed birds. Any associations identified will be further investigated in subsequent research, contributing to a deeper understanding of APEC dynamics.

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2. MATERIALS AND METHODS

We measured associations among resistance and susceptibility to antibiotics, virulence gene and phylogenetic groups of APEC isolates that we obtained in previous studies [20, 21], assessed the role of virulence genes and phylogenetic groups [20] as predictors of phenotypic and genotypic resistance to various antibiotics. The methods of sample collection, sample type, isolation, identification, stocking, phenotypic resistance, DNA extraction, molecular characterization based on phylogenetic groups, and VAGs, ARGs of APEC isolates well-described in previous studies [20, 21].

Statistical analyses were conducted using R-Studio [22]. Logistic regression was used to identify associations among resistance and susceptibility to antibiotic agents, virulence and phylogenetic groups of APEC isolates. To ensure an adequate amount of variability in the data, only antimicrobials with resistance rates in the range 5%–95% across the isolates were considered for analysis. Binary logistic regression was used to access the role of virulence genes, ARG and phylogenetic group as predictors of phenotypic and genotypic resistance to various antibiotic agents. Pairwise comparisons between resistance to an individual antimicrobial (yes/no) and the presence of an individual virulence-associated gene (yes/no) were carried out using univariable logistic regression models. A significance level of $p \leq 0.05$ (Wald test) was employed to indicate a statistically significant association [23]. In each model, the dependent variable was the resistance of an isolate to a specific antimicrobial (yes/no), while the independent variables were the ARG and VAGs, deemed significant at the univariable stage.

3. RESULTS

3.1 Association of antibiotic resistance with different phylogenetic groups

Logistic regression analysis revealed no significant association between resistance or susceptibility to antibiotic agents and the phylogenetic origins of APEC isolates, except for neomycin, ceftriaxone, chloramphenicol, cotrimoxazole and doxycycline (Tables 1 and 2). The resistance patterns varied among eight phylotypes, with the highest in phylotype F, followed by A, E, D, B1 and C (Table 1). APEC isolates from all phylogenetic group were completely resistant to ampicillin (100%) and most sensitive to azithromycin (3.1%), amikacin (7.2%) and colistin (4.7%). Resistance to amikacin and azithromycin were not identified in E. clade phylogenetic group.

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Table 1: Rate of occurrence of phenotypic antibiotic resistance in various phylogenetic groups in avian pathogenic *Escherichia coli* isolated from commercial broiler, layer and broiler and layer breeder in Nepal

Phenotypic antibiotic resistance	A (*n* = 66)	B1 (n = 96)	B2 (n = 58)	C (n = 29)	D (n = 22)	E (n = 91)	F (n = 22)	E. clade (n = 15)	Unknown (n = 88)	Grand Total
Neomycin (NE, 10µg)	65 (98.5)	**89 (92.7)**	45 (77.6)	25 (86.2)	19 (86.4)	87 (95.6)	22 (95.6)	12 (80)	84 (94.3)	447 (91.8)
Gentamicin (GEN, 10µg)	34 (51.5)	41 (42.7)	28 (48.3)	17 (58.6)	10 (45.5)	45 (49.5)	12 (49.5)	4 (26.7)	**54 (61.4)**	245 (50.3)
Amikacin (AK, 30)	13 (19.7)	12 (12.5)	9 (15.5)	6 (20.7)	1 (4.5)	17 (18.7)	2 (18.7)	0	12 (13.6)	72 (14.8)
Ampicillin (AP, 10µg)	66 (100)	96 (100)	56 (96.6)	29 (100)	22 (100)	91 (100)	22 (100)	15 (100)	88 (100)	485 (99.6)
Ceftriaxone (CRO, 30 µg)	28 (42.4)	**42 (43.8)**	25 (43.1)	6 (20.7)	7 (31.8)	37 (40.7)	10 (40.7)	3 (20)	**25 (28.4)**	183 (37.6)
Trimethoprim/ Sulphamethoxazole (TS 250µg)	46 (69.7)	66 (68.8)	33 (56.9)	18 (62.1)	**10 (45.5)**	62 (68.1)	13 (68.1)	9 (60)	59 (67)	316 (64.9)
Doxycycline (DXT, 30µg)	57 (86.4)	88 (91.7)	51 (87.9)	27 (93.1)	20 (90.9)	82 (90.1)	22 (90.1)	**10 (66.7)**	81 (92)	438 (89.9)
Tetracycline (T30 µg)	53 (80.3)	84 (87.5)	46 (79.3)	22 (75.9)	20 (90.9)	77 (84.6)	22 (84.6)	10 (66.7)	72 (81.8)	406 (83.4)
Azithromycin (ATH, 15 µg)	2 (3.0)	1 (1)	2 (3.4)	1 (3.4)	1 (4.5)	4 (4.4)	2 (4.4)	0	2 (2.3)	15 (3.1)
Chloramphenicol (C, 30µg)	32 (48.5)	**56 (58.3)**	18 (31)	10 (34.5)	11 (50)	42 (46.2)	10 (46.2)	5 (33.3)	**27 (30.7)**	211 (43.3)
Ciprofloxacin (CIP, 5µg)	62 (93.9)	92 (95.8)	54 (93.1)	26 (89.7)	21 (95.5)	87 (95.6)	21 (95.6)	14 (93.3)	85 (96.6)	462 (94.9)
Enrofloxacin (ENF, 10 µg)	64 (97)	91 (94.8)	54 (93.1)	26 (89.7)	22 (100)	84 (92.3)	20 (92.3)	13(86.7)	84 (95.5)	458 (94.1)
Levofloxacin (LEV, 5µg)	55 (83.3)	86 (89.6)	53 (91.4)	**27 (93.1)**	19 (86.4)	78 (85.7)	78 (85.7)	13 (86.7)	13 (93.2)	434 (89.1)
Colistin (CO, 0.8mg)	18 (27.3)	27 (28.1)	21 (36.2)	8 (27.6)	8 (36.4)	26 (28.6)	26 (28.6)	8 (53.3)	8 (35.2)	156 (32)

Notes: Bold n (%): Binary logistic regression analysis showed significant association ($p \leq 0.05$). Figures in parenthesis indicate the number of phenotypic antibiotic resistance.

The highly resistant isolates to neomycin (92.7%) (OR = 1.76, p = 0.046), ceftriaxone (43.8%) (OR = 1.71, p = 0.02), and chloramphenicol (58.3%) (OR = 2.1, p = <0.001) were associated with phylogenetic group B1. Phylogenetic group C (OR = 0.38, p = 0.0018), phylogenetic group D (OR = 0.37, p = 0.025), phylogenetic group E clade (OR = 0.25, p = 0.009) origins were associated with levofloxacin (93.1%), cotrimoxazole (45.5%) and doxycline (66.7%) susceptibility. The majority of highly resistant gentamicin, sensitive to ceftriaxone and chloramphenicol isolates were significantly associated with an unknown phylogenetic group, absent from A, B1, B2, C, D, E, F and *Escherichia* cryptic clade (*E.* clade) phylogenetic groups. Overall, the highest resistant isolates were placed with phylogenetic group E (19.7%) followed by phylogenetic group B1 (19.1%) with azithromycin, amikacin and gentamicin showing the highest resistance. Isolates resistant to rest of the antibiotics were mostly belonging to the phylogenetic group B1 (Table 2), which was significantly higher than other phylogenetic group.

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Table 2: Binary logistic regression analysis of antibiotic resistance as predictor of phylogenetic origins in avian pathogenic *Escherichia coli* from chickens

Antibiotic Resistance	Phylogenetic origins																	
	A (*n* = 66)		B1 (n = 96)		B2 (n = 58)		C (n = 29)		D (n = 22)		E (n = 91)		F (n = 22)		E. clade (n = 22)		Unknown (n = 88)	
	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p
Neomycin (NE, 10µg)	0.98	>0.9	**1.76**	**0.046**	0.79	0.4	0.96	>0.9	0.63	0.3	1.38	0.2	1.26	0.7	0.54	0.3	0.66	0.094
Gentamicin (GEN, 10µg)	1.04	0.9	0.67	0.1	1.34	0.3	1.52	0.3	1.11	0.8	0.66	0.09	1.11	0.8	0.57	0.3	**1.58**	**0.053**
Amikacin (AK, 30)	0.69	0.3	0.74	0.4	0.83	0.6	1.83	0.2	0.45	0.3	1.5	0.2	1.78	0.2	0.32	0.3	1.13	0.7
Ampicillin (AP, 10µg)	0.31	0.3	-	>0.9	0.27	0.3	-	>0.9	-	>0.9	-	>0.9	-	>0.9	-	>0.9	0.44	0.5
Ceftriaxone (CRO, 30 µg)	1.15	0.6	**1.71**	**0.02**	1.6	0.094	0.53	0.2	1.21	0.7	0.93	0.8	1.47	0.4	0.86	0.8	**0.35**	**<0.001**
Trimethoprim/ Sulphamethoxazole (TS 250µg)	1.34	0.3	1.09	0.7	0.77	0.4	0.91	0.8	**0.37**	**0.025**	1.17	0.5	0.8	0.6	0.63	0.4	1.25	0.4
Doxycycline (DXT, 30µg)	0.75	0.4	1.44	0.3	1.5	0.3	1.11	0.8	0.6	0.3	1.33	0.4	0.77	0.6	**0.25**	**0.009**	0.95	0.9
Tetracycline (T30 µg)	0.79	0.5	1.52	0.2	0.93	0.9	0.68	0.4	0.99	>0.9	0.95	0.9	-	>0.9	0.43	0.13	0.9	0.7
Azithromycin (ATH, 15 µg)	0.79	0.8	0.23	0.2	0.43	0.4	0.93	>0.9	1.25	0.8	2.26	0.11	2.81	0.2	1.91	0.5	0.9	0.9
Chloramphenicol (C, 30µg)	1.05	0.9	**2.18**	**<0.001**	0.63	0.11	0.82	0.6	0.98	>0.9	1.19	0.5	1.18	0.7	0.42	0.14	**0.58**	**0.026**
Ciprofloxacin (CIP, 5µg)	0.84	0.7	1.66	0.3	0.87	0.8	0.46	0.13	0.63	0.5	1.55	0.3	2.19	0.4	1.44	0.7	0.75	0.4
Enrofloxacin (ENF, 10 µg)	0.83	0.8	1.32	0.7	1.16	0.8	0.52	0.4	-	>0.9	0.63	0.4	0.85	0.9	-	>0.9	1.18	0.8
Levofloxacin (LEV, 5µg)	1.08	0.8	1.32	0.4	1.39	0.4	**0.38**	**0.018**	0.55	0.2	1.11	0.7	0.96	>0.9	0.85	0.8	1.05	0.9
Colistin (CO, 0.8mg)	2.38	0.08	1.14	0.8	1.12	0.9	0	>0.9	0	>0.9	0	>0.9	2.11	0.3	0	>0.9	2.07	0.12

Notes: OR odds ratio, p p-value; Bold p: Significant association ($p \leq 0.05$) was shown by binary logistic regression analysis; When $p \leq 0.05$, then $OR > 1$ means that phylogenetic group is introduced as predictor of that antibiotic resistance, but $OR < 1$ means that phylogenetic group is a predictor of antibiotic sensitivity

3.2 Association of virulence associated gene with different phylogenetic groups

Among the 57 genes investigated, the number of genes detected varied, ranging from as few as 8 (3 isolates, 0.6%) to as many as 26 (five isolates, 1%) with the most prevalent harboring 16 and 20 genes (13.1% each) per isolate. We found 48 VAGs (out of 57 VAGs tested) in eight phylogenetic groups, except *ironEC* and *aerJ* with phylogenetic group C and *aerJ* with phylogenetic group F, which were completely absent. Among 57 investigated virulence genes *fimH*, *iss*[a], *traT*[a], *sit chr. ironEC*, *traT*[b], *kpsII*, *iss*[b], *cvi.cva*, *iucD*, *aerJ*, and *ompT* were the top 10 genes detected in APEC isolates. The carriage of most VAGs was significantly associated with particular phylogenetic groups. However, *fimH (*99.2%) was an exception, as

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it was the most prevalent and associated equally with all phylogenetic groups. This pattern was also observed with *iss*[a], *traT*[a], *sit.chr*., *kpsII*, *iss*[b], and *cvi.cva*. The majority of the VAGs (*kpsIII*, *ireA*, *aerJ*, *ompT*, *papGII*, *etsB*, *colB*, *eitA iucD*, *sit. ep.* and *ompA*) were significantly associated with phylogenetic group D and they were absent from other groups (Table 3 and 4). The carriage of *ironEC*, *papGI*, *papGII.III*, *eitB* and *traT*[b] were significantly associated with phylogenetic group E. The carriage of *malX*, *fyeA*, *papGIII,* and *iroN* were significantly associated with the E clade. The carriage of *colM* and *iha* were significantly associated with phylogenetic group A. The carriage of *papC*[a], *cvaC*, *tsh, sit. ep.* and *irp2* were significantly associated with phylogenetic group C. The carriage of *sfa.foc* was significantly associated with B2 compared to other groups. Phylogenetic group E. clade isolates showed reduced carriage of *kpsII* and *iss*[a] compared to the other groups. The occurrence of the *traT*[a] and *sit. chr.* gene in group B1 was significantly less than in other groups. The occurrence of the *iss*[b] gene in group A, *malX* gene in phylogroup B2 and *cvi.cva* gene with phylogenetic group D were significantly less than in the other groups (Table 3 and 4).

Table 3: Occurrence of virulence genes in various phylogenetic groups of avian pathogenic *Escherichia coli* from chickens

Gene	A (*n* = 66)	B1 (n = 96)	B2 (n = 58)	C (n = 29)	D (n = 22)	E (n = 91)	F (n = 22)	E. Clade (n = 15)	Unknown (n = 84)	Grand Total
fimH	66 (100)	96 (100)	58 (100)	29 (100)	22 (100)	91 (100)	22 (100)	15 (100)	84 (100)	483 (99.2)
iss[a]	61 (92.4)	90 (93.8)	53 (91.4)	29 (100)	22 (100)	91 (100)	22 (100)	5 (33.3)	73 (86.9)	446 (91.6)
traT[a]	63 (95.5)	71 (74)	58 (100)	29 (100)	17 (77.3)	82 (90.1)	22 (100)	15 (100)	80 (95.2)	437 (89.7)
sit chr.	54 (81.8)	75 (78.1)	47 (81)	29 (100)	22 (100)	75 (82.4)	22 (100)	12 (80)	78 (92.9)	414 (85)
ironEC	66 (100)	86 (89.6)	45 (77.6)	0	22 (100)	87 (95.6)	22 (100)	15 (100)	68 (81)	411 (84.4)
traT[b]	36 (54.5)	80 (83.3)	50 (86.2)	29 (100)	19 (86.4)	88 (96.7)	22 (100)	9 (60)	67 (79.8)	400 (82.1)
kpsII	41 (62.1)	73 (76)	49 (84.5)	29 (100)	22 (100)	74 (81.3)	10 (45.5)	3 (20)	72 (85.7)	373 (76.6)
iss[b]	**33 (50)**	55 (57.3)	45 (77.6)	29 (100)	19 (86.4)	71 (78)	22 (100)	12 (80)	75 (89.3)	361 (74.1)
cvi.cva	52 (78.8)	60 (62.5)	36 (62.1)	25 (86.2)	9 (40.9)	73 (80.2)	22 (**100**)	12 (80)	65 (77.4)	354 (72.7)
iucD	44 (66.7)	55 (57.3)	36 (62.1)	25 (86.2)	22 (100)	70 (76.9)	22 (100)	9 (60)	65 (77.4)	348 (71.5)
aerJ	52 (78.8)	85 (88.5)	40 (69)	0	22 (100)	61 (67)	0	9 (60)	72 (85.7)	341 (70)
ompT	37 (56.1)	65 (67.7)	50 (86.2)	29 (100)	22 (100)	**53 (58.2)**	22 (100)	9 (60)	53 (63.1)	340 (69.8)

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Table 4: Binary logistic regression analysis of virulence associated genes as predictor of phylogenetic groups in avian pathogenic *Escherichia coli* from chickens

Characteristics	Phylogenetic groups														
	B1		E		A		B2		C		D		F	E CLADE	
	OR	*p*	*p*	OR	*p*	OR	*p*	OR	*p*	OR	*p*	OR	*p*	OR	*p*
MalX	0	>0.9	0.3	0	>0.9	**0.13**	**0.045**	1.74	0.3	2.35	0.1		>0.9	**5.89**	**0.001**
fimH		>0.9	>0.9		>0.9		>0.9		>0.9	0.14	0.1		>0.9		>0.9
kpsIII	1.37	0.3	>0.9	1.4	0.3	**3**	**<0.001**	0	>0.9	**6.14**	**<0.001**	0	>0.9	1.84	0.3
ireA	0.86	0.5	0.5	0.61	0.063	**3.09**	**<0.001**	1.92	0.11	**19.8**	**0.004**	1	>0.9	1.25	0.7
fyuA	0.66	0.14	0.3	1.11	0.7	**2.09**	**0.013**	0	>0.9	**4.13**	**0.001**	0	>0.9	**4.66**	**0.004**
ironEC	1.77	0.11	**0.003**		>0.9	0.64	0.2	0	>0.9	4.24	0.2		>0.9		>0.9
sfa.foc	1.01	>0.9	0.8	0	>0.9	**2.98**	**0.009**	0	>0.9	2.02	0.3	0	>0.9	0	>0.9
aerJ	**3.71**	**<0.001**	0.5	1.7	0.1	1.08	0.8	0	>0.9	**10**	**0.025**	0	>0.9	0.63	0.4
papGIII	**0.32**	**0.006**	**0.022**	0.97	>0.9	0.55	0.2	0	>0.9	0	>0.9		>0.9	**3.42**	**0.023**
ompT	0.9	0.7	**0.008**	**0.5**	**0.01**	**2.86**	**0.008**		>0.9	**10.1**	**0.024**		>0.9	0.64	0.4
papGI	**0.47**	**0.003**	**<0.001**	1.46	0.2	0.61	0.1	0	>0.9	1.14	0.8		>0.9	0	>0.9
kpsII	0.98	>0.9	0.2	**0.44**	**0.003**	1.68	0.2		>0.9	7.08	0.057	**0.23**	**0.001**	**0.07**	**<0.001**
papC[a]	1.11	0.6	**0.005**	1.29	0.3	**0.57**	**0.048**	**2.38**	**0.041**	0.65	0.3	0	>0.9	0	>0.9
cvaC	**0.46**	**0.002**	**0.02**	1.07	0.8	1.63	0.087	**10.9**	**<0.001**	**2.5**	**0.036**	1.3	0.6	1.03	>0.9
traT[a]	**0.2**	**<0.001**	0.9	2.64	0.11		>0.9		>0.9	**0.3**	**0.015**		>0.9		>0.9
papGII	0	>0.9	0.11	0	>0.9	**2.53**	**0.042**	0	>0.9	**4.88**	**0.004**	0	>0.9	0	>0.9
papGII.III	0.59	0.3	**<0.001**	0.73	0.6	0	>0.9	0	>0.9	0	>0.9	0	>0.9	0	>0.9
iha	0.44	0.09	0.062	**2.38**	**0.017**	0.88	0.8	2.01	0.2	0	>0.9	0	>0.9	0	>0.9
iss[a]	1.5	0.4	>0.9	1.14	0.8	0.93	0.9		>0.9	2.08	0.5		>0.9	**0.04**	**<0.001**
etsB	**2.46**	**<0.001**	**0.008**	0.82	0.5	1.07	0.8	0	>0.9	**12.8**	**0.013**	0	>0.9	0.81	0.7
colM	0.59	0.055	0.2	**1.77**	**0.037**	1.33	0.3	0	>0.9	0	>0.9		>0.9	1.7	0.3
colB	**2.09**	**0.003**	**0.004**	1.03	>0.9	0.88	0.6	0	>0.9	**15.8**	**0.007**	0	>0.9	**0.16**	**0.005**
eitB	0.81	0.4	**<0.001**	1.18	0.5	**0.3**	**<0.001**	0	>0.9	0	>0.9		>0.9	1.55	0.4
eitA	**0.54**	**0.008**	0.3	1.44	0.2	**2.21**	**0.02**	0	>0.9	**13.1**	**0.012**		>0.9	0.83	0.7
tsh	**0.58**	**0.04**	0.4	1.62	0.076	**0.44**	**0.023**	**6.48**	**<0.001**	0	>0.9		>0.9	0.54	0.3
iucD	**0.43**	**<0.001**	0.2	0.77	0.4	0.69	0.2	2.61	0.08	**9.31**	**0.03**		>0.9	0.59	0.3
cvi.cva	**0.56**	**0.016**	0.076	1.46	0.2	0.59	0.071	2.45	0.1	**0.22**	**<0.001**		>0.9	1.52	0.5
irp2	**0.45**	**0.001**	**<0.001**	1.49	0.14	**2.33**	**0.004**	**3.3**	**0.005**	0.9	0.8		>0.9	1.79	0.3
iss[b]	**0.38**	**<0.001**	0.3	**0.28**	**<0.001**	1.18	0.6		>0.9	1.69	0.3		>0.9	1.41	0.6
sit.ep.	**0.4**	**0.021**	0.3	1.17	0.6	1.88	0.061	**2.49**	**0.03**	**11.7**	**<0.001**	0	>0.9	0	>0.9
ompA	**1.8**	**0.011**	**<0.001**	1.49	0.14	**2.28**	**0.004**	0.85	0.7	**10.1**	**<0.001**	0	>0.9	0.96	>0.9
iroN	**3.01**	**<0.001**	0.3	0.66	0.3	1.74	0.092	0	>0.9	0.93	0.9	0	>0.9	**7.24**	**<0.001**
sit.chr	**0.56**	**0.042**	0.4	0.76	0.4	0.69	0.3		>0.9	4.04	0.2		>0.9	0.7	0.6
traT[b]	1.13	0.7	**<0.001**	**0.19**	**<0.001**	1.35	0.5		>0.9	1.03	>0.9		>0.9	**0.31**	**0.031**

Notes: OR: odds ratio, p: p-value; Bold p: Significant association ($p \leq 0.05$) was shown by binary logistic regression analysis; When $p \leq 0.05$, then OR > 1 means that VAGs is introduced as predictor of that phylogenetic groups, but OR < 1 means that VAGs is a predictor of other phylogenetic groups

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3.3 Associations between virulence genes harbored and antibiotic resistant genes

The occurrence of various VAGs in antibiotic-resistant isolates was investigated separately for each antibiotic agent. Binary logistic regression analysis revealed a significantly high number of VAG in highly resistant isolates showing resistance to ciprofloxacin and tetracycline antibiotic agents (Table 6). Genes such as *malX*, *cvaC, iroN, iha, sfa.foc, kpsIII* and *iucD* were significantly more prevalent in isolates sensitive to different classes of antibiotic agents compared to resistant isolates. In contrast, genes like *ompA*, *traT*^{b}, *iss*^{b}, *cvi.cva*, *eitB*, *colB*, *etsB*, *traT*^{a}, *kpsII*, *papGI*, *ompT*, *colM*, *irp2*, *tsh*, *papGII.III*, *papC*^{a}, *fyuA*, *ironEC* and *aerJ* genes were notably more abundant in isolates resistant to various antibiotic agents (Table 6). Certain genes, including *papA*, *papEF*, *cnf-1*, *bmaE*, *sfa-foc*, *papGI*^{a}, *fliC papGII*, *afa*, *sfaS*, *hlyF* and *gimB* were not observed in azithromycin resistant isolates, while *afa* gene was absent in gentamicin resistant isolates and *cnf-1* gene absent in ceftriaxone-resistant isolates (Table 5). Simple pairwise comparisons between resistance to an individual antimicrobial and the presence of an individual VAG are shown in Table 6. In general, individual genes exhibited positive association with ciprofloxacin, tetracycline, gentamicin, neomycin, doxycycline, levofloxacin, amikacin, and cotrimoxazole resistance, a negative association with ceftriaxone, and no association with chloramphenicol and enrofloxacin resistance. The *ireA*, *fyuA*, *ompT*, *papGI*, *papC*^{a}, *traT*^{a}, *colB*, and *eitB* genes were associated with ciprofloxacin resistance. The *ironEC*, *aerJ*, *etsB, kpsII*, *iss*^{a} and *traT*^{b} genes were associated with tetracycline resistance. Positive associations were observed between tetracycline resistance and the presence of *papGI* (OR = 1.76, p = 0.031) and *traT*^{b} (OR = 1.8, p = 0.04), *ironEC*, *aerJ*, *kpsII*, *etsB*, *iss*^{a}. The *ompA* gene was associated with levofloxacin resistance (OR = 3.35, p = 0.014,) and *colM* gene was associated with neomycin resistance (OR = 3.36, p = 0.05). Gentamicin resistance showed positive association with the presence of *papGII.III* (OR = 2.6, p = 0.01) and *tsh* (OR = 1.72, p = 0.006) compared to other VAGs. Amikacin resistance was positively associated with the presence of *cvi.cva* (OR = 1.7, p = 0.035). Likewise, doxycycline resistance was positively associated with the presence of *irp2* (OR = 1.7, p = 0.035). Cotrimoxazole resistance was positively associated but to a lesser extent than other resistances. The *fimH*, *papGII*, *papGIII*, *iss*^{a}, *eitA*, *sit.ep.*, *sit chro* genes showed no significant association with any resistance or susceptibility.

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Table 5: Frequency of different virulence genes in antibiotic-resistant avian pathogenic *Escherichia coli* isolates

Antibiotics agents	R or S (n)	Virulence genes n (%)											
		fimH	*iss*[a]	*traT*[a]	*sit chr.*	*ironEC*	*traT*[b]	*kpsII*	*iss*[b]	*cvi.cva*	*iucD*	*aerJ*	*ompT*
Neomycin (NE, 10µg)	**R** (447)	443 (99.1)	413 (92.4)	398 (89)	378 (84.6)	379 (84.8)	363 (81.2)	342 (76.5)	328 (73.4)	324 (72.5)	318 (71.1)	313 (70)	308 (68.9)
	S (40)	40 (100)	33 (82.5)	39 (97.5)	36 (90)	32 (80)	37 (92.5)	31 (77.5)	33 (82.5)	30 (75)	30 (75)	28 (70)	32 (80)
Gentamicin (GEN, 10µg)	**R** (245)	242 (98.8)	225 (91.8)	218 (98)	206 (84.1)	198 (80.8)	205 (83.7)	188 (76.7)	179 (73.1)	187 (76.3)	182 (74.3)	167 (68.2)	171 (69.8)
	S (242)	241 (99.6)	221 (91.3)	219 (90.5)	208 (86)	213 (88)	195 (80.6)	185 (76.4)	182 (75.2)	167 (69)	166 (68.6)	174 (71.9)	169 (69.8)
Amikacin (AK, 30)	**R** (73)	72 (98.6)	68 (93.2)	63 (86.3)	56 (76.7)	60 (82.2)	60 (82.2)	49 (67.1)	47 (64.4)	59 (80.8)	53 (72.6)	47 (64.4)	48 (65.8)
	S (414)	411 (99.3)	378 (91.3)	374 (90.3)	358 (86.5)	351 (84.8)	340 (82.1)	324 (78.3)	314 (75.8)	295 (71.3)	295 (71.3)	294 (71)	292 (70.5)
Ampicillin (AP, 10µg)	**R** (487)	483 (99.2)	446 (91.6)	437 (89.7)	414 (85)	411 (84.4)	400 (82.1)	373 (76.6)	361 (74.1)	354 (72.7)	348 (71.5)	341 (70)	340 (69.8)
Ceftriaxone (CRO, 30 µg)	**R** (183)	181 (98.9)	173 (94.5)	157 (85.8)	150 (82)	159 (86.9)	153 (83.6)	139 (76)	134 (73.2)	135 (73.8)	132 (72.5)	125 (68.3)	129 (70.5)
	S (304)	302 (99.3)	273 (89.8)	280 (92.1)	264 (86.8)	252 (82.9)	247 (81.3)	234 (77)	227 (74.7)	219 (72)	216 (71.1)	216 (71.1)	211 (69.4)
Trimethoprim/ Sulphamethoxazole (TS 250µg)	**R** (316)	312 (98.7)	291 (92.1)	291 (92.1)	268 (84.8)	268 (84.8)	264 (83.5)	243 (76.9)	239 (75.6)	230 (72.8)	226 (71.5)	215 (68)	213 (67.4)
	S (171)	171 (100)	155 (90.6)	146 (85.4)	146 (85.4)	143 (83.6)	136 (79.5)	130 (76)	122 (71.3)	124 (72.5)	122 (71.3)	126 (73.7)	127 (74.3)
Doxycycline (DXT, 30µg)	**R** (438)	434 (99.1)	402 (91.8)	392 (89.5)	370 (84.5)	367 (83.8)	364 (83.1)	334 (76.3)	323 (73.7)	315 (71.9)	314 (71.7)	307 (70.1)	308 (70.3)
	S (49)	49 (100)	44 (89.8)	45 (91.8)	44 (89.8)	44 (89.8)	36 (73.5)	39 (79.6)	38 (77.6)	39 (79.6)	34 (69.4)	34 (69.4)	32 (65.3)
Tetracycline (T30 µg)	**R** (406)	403 (99.3)	373 (91.9)	364 (89.7)	349 (86)	348 (85.7)	340 (83.7)	313 (77.1)	302 (74.4)	291 (71.7)	290 (71.4)	291 (71.7)	283 (69.7)
	S (81)	80 (98.8)	73 (90.1)	73 (90.1)	65 (80.2)	63 (77.8)	60 (73.1)	60 (74.1)	59 (72.8)	63 (77.8)	58 (71.6)	50 (61.7)	57 (70.4)
Azithromycin (ATH, 15 µg)	**R** (15)	15 (100)	14 (93.3)	14 (93.3)	13 (86.7)	13 (86.7)	14 (93.3)	11 (73.3)	13 (86.7)	13 (89.7)	13 (89.7)	10 (66.7)	8 (53.3)
	S (472)	468 (99.2)	432 (91.5)	423 (89.6)	401 (85)	398 (84.3)	386 (81.8)	362 (76.7)	348 (73.7)	341 (72.2)	335 (71)	331 (70.1)	332 (70.3)
Chloramphenicol (C, 30µg)	**R** (210)	208 (99)	191 (91)	185 (88.1)	177 (84.3)	183 (87.1)	174 (82.9)	154 (73.3)	140 (66.7)	155 (73.8)	142 (67.6)	162 (77.1)	153 (72.9)
	S (277)	275 (99.3)	255 (92.1)	252 (91)	237 (85.6)	228 (82.3)	226 (81.6)	219 (79.1)	221 (79.8)	199 (71.8)	206 (74.4)	179 (64.6)	187 (67.5)
Ciprofloxacin (CIP, 5µg)	**R** (462)	458 (99.1)	422 (91.3)	415 (89.8)	391 (84.6)	390 (84.4)	379 (82)	353 (76.4)	341 (73.8)	334 (72.3)	327 (70.8)	324 (70.1)	322 (69.7)
	S (25)	25 (100)	24 (96)	22 (88)	23 (92)	21 (84)	21 (84)	20 (80)	20 (80)	20 (80)	21 (84)	17 (68)	18 (72)
Enrofloxacin (ENF, 10 µg)	**R** (454)	421(92.7)	410(90.3)	386(85)	374(82.4)	352(77.5)	337(74.2)	327(72)	324(71.4)	389(85.7)	322(70.9)	331(72.9)	147(32.4)
	S (29)	25 (86.2)	27 (93.1)	25 (86.2)	26 (89.7)	21 (72.4)	24 (82.8)	21 (72.4)	17 (58.6)	25 (86.2)	18 (62.1)	23 (79.3)	9 (310)
Levofloxacin (LEV, 5µg)	**R** (434)	430 (99.1)	397 (91.5)	388 (89.4)	366 (84.3)	363 (83.6)	357 (82.3)	334 (77)	318 (73.3)	314 (72.4)	308 (71)	303 (69.8)	307 (70.7)
	S (53)	53 (100)	49 (92.5)	49 (92.5)	48 (90.6)	48 (90.6)	43 (81.1)	39 (73.6)	43 (81.1)	40 (75.5)	40 (75.5)	38 (71.7)	33 (62.3)
Colistin (CO, 0.8mg)	**R** (156)	155 (99.4)	139 (89.1)	145 (92.9)	133 (85.3)	131 (84)	130 (83.3)	116 (74.4)	117 (75)	114 (73.1)	106 (67.9)	108 (69.2)	110 (70.5)
	S (331)	328 (99.1)	307 (92.7)	292 (88.2)	281 (84.9)	280 (84.6)	270 (81.6)	257 (77.6)	244 (73.7)	240 (72.5)	242 (73.1)	233 (70.4)	230 (69.5)
		483	446	437	414	411	400	373	361	354	348	341	340

Notes: Figure in parentheses indicates the percent. R: Resistant, S: Sensitive

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Table 6: Binary logistic regression analysis of virulence-associated genes as predictor of resistance to various antibiotic agents in avian pathogenic *Escherichia coli*

	Neomycin (NE, 10µg)		Gentamicin (GEN, 10µg)		Amikacin (AK, 30)		Ceftriaxone (CRO, 30 µg)		Cotrimoxazole (TS 250µg)		Doxycycline (DXT, 30µg)		Tetracycline (T30 µg)		Chloramphenicol (C, 30µg)		Ciprofloxacin (CIP, 5µg)		Enrofloxacin (ENF, 10 µg)		Levofloxacin (LEV, 5µg)	
	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p
MalX	0.7	0.5	0.8	0.4	1.1	0.7	1.6	0.1	0.8	0.5	0.8	0.6	**0.75**	**<0.001**	0.75		1.25	0.3	0.49	0.5	0.7	0.5
fimH	0	>0.9	0.3	0.3	0.3	0.3	0.8	0.8	0	>0.9	0	>0.9	1.68	>0.9	0.96			>0.9	0	>0.9	0	>0.9
kpsIII	0.56	0.2	**0.6**	**0.026**	**0.5**	**0.028**	1.4	0.2	**0.6**	**0.044**	0.7	0.3	1.2	0.06	1.11		0.91	0.1	-	>0.9	1.2	0.7
ireA	0.49	0.1	0.9	0.4	1.1	0.7	1.1	0.8	1.1	0.7	1.2	0.6	1.14	0.3	1.12		**1**	**<0.001**	0.8	0.8	1.2	0.7
fyuA	0.56	0.2	0.9	0.5	0.9	0.7	0.9	0.4	0.8	0.4	0.9	0.8	**1.05**	**0.019**	0.92		**1.55**	**<0.001**	1.37	0.8	1	>0.9
ironEC	1.25	0.7	**0.6**	**0.03**	0.8	0.5	1.4	0.2	1.2	0.4	0.5	0.2	**1.71**	**0.001**	1.31		**1.21**	**0.001**	3.68	0.2	0.7	0.5
sfa.foc	0.97	>0.9	0.8	0.6	1.8	0.11	1	>0.9	0.8	0.6	1.1	0.9	0.78	0.3	0.76		**0.34**	**0.002**	0.3	0.3	2.8	0.3
aerJ	1.18	0.7	0.8	0.4	**0.7**	**0.054**	1	0.8	0.8	0.4	0.8	0.6	**1.57**	**<0.001**	1.79		**0.87**	**0.003**	3.56	0.2	0.9	0.9
papGIII	0.9	0.8	1	0.9	1.4	0.2	1.2	0.4	1.3	0.4	1	0.9	0.89	0.074	0.71		2.08	0.7	0.82	0.9	0.8	0.6
ompT	0.39	0.084	1	>0.9	0.8	0.3	1.2	0.3	0.7	0.11	1.5	0.2	**0.97**	**<0.001**	1.18		**1.33**	**0.009**	0	>0.9	1.6	0.2
papGI	2.01	0.12	1.1	0.5	1.5	0.075	0.9	0.6	**1.5**	**0.047**	1.3	0.5	**1.76**	**<0.001**	0.82		**1.83**	**<0.001**	2.74	0.4	0.8	0.5
kpsII	0.93	0.9	1	>0.9	0.8	0.2	0.8	0.3	1	>0.9	0.8	0.6	**1.18**	**0.017**	0.79		1.23	>0.9	2.2	0.4	1.3	0.5
papC[a]	1.26	0.6	**1.5**	**0.021**	1.2	0.4	**0.7**	**0.038**	1.1	0.7	1	>0.9	**1.38**	**<0.001**	0.9		**2.05**	**0.003**	0.77	0.8	0.9	0.7
cvaC	0.94	0.9	1	>0.9	0.9	0.6	**0.7**	**0.035**	0.8	0.14	0.9	0.7	0.88	0.5	1.18		0.78	0.4	0.43	0.4	1	>0.9
traT[a]	0	>0.9	0.9	0.6	0.8	0.5	0.6	0.1	1.7	0.085	0.8	0.7	0.95	>0.9	0.88		**1.98**	**0.005**	2.21	0.5	0.8	0.7
papGII	0.5	0.3	1.8	0.15	0.7	0.5	0.7	0.3	1.2	0.7	0.9	0.9	1	>0.9	0.5			>0.9	0.26	0.2	0.7	0.5
papGII.III	-	>0.9	**2.6**	**0.01**	1.4	0.4	0.9	0.7	1.1	0.8	0.9	0.8	1.07	0.092	1.04			0.3	-	>0.9	0.9	0.9
iha	0.61	0.4	1.1	0.8	1	>0.9	0.9	0.6	0.9	0.7	0.9	0.8	**0.64**	**<0.001**	1.04		1.1	0.15	0.43	0.5	0.6	0.4
iss[a]	2.68	0.061	1.1	0.8	0.9	0.9	1.5	0.3	1.3	0.4	1.4	0.5	1.24	0.3	0.91			0.15	0	>0.9	1	>0.9
etsB	0.51	0.15	0.7	0.11	0.8	0.4	0.9	0.6	1	>0.9	1.1	0.7	**1.4**	**0.005**	1.46		1.54	0.01	7.48	0.073	1	0.9
colM	**3.36**	**0.051**	1.2	0.3	1	>0.9	1.3	0.3	1	>0.9	0.8	0.5	0.82	>0.9	0.72		1.07	0.2	0.26	0.14	1	>0.9
colB	0.87	0.7	0.8	0.14	1.1	0.8	1	>0.9	1.1	0.8	1.2	0.5	**1.49**	**0.027**	1.55		**1.82**	**0.034**	6.09	0.11	1	>0.9
eitB	2.04	0.09	1.3	0.2	**1.6**	**0.021**	1	>0.9	**1.7**	**0.005**	0.8	0.6	**1.13**	**<0.001**	0.76		**1.74**	**0.008**	0.65	0.6	0.7	0.3
eitA	1.47	0.3	0.9	0.4	1.1	0.6	1.2	0.4	1.1	0.8	1	>0.9	1.07	0.4	1.35		1.03	0.5	1.2	0.8	1.2	0.6
tsh	2.75	0.066	**1.7**	**0.006**	**2.1**	**<0.001**	1.3	0.2	**1.5**	**0.048**	1.9	0.088	1.19	>0.9	1.12		0.8	0.3	0.3	0.2	1.2	0.7
lucD	0.7	0.5	1.3	0.2	1.4	0.2	1.1	0.6	1.2	0.5	1	>0.9	**0.99**	**<0.001**	0.86		0.55	0.11	0.62	0.7	1	>0.9
cvi.cva	1.35	0.5	1.5	0.071	**1.7**	**0.035**	1.1	0.7	1.1	0.8	0.6	0.3	0.72	0.7	1.15		0.59	0.024	0.66	0.7	0.7	0.3
irp2	1.07	0.9	**1.5**	**0.04**	1.4	0.077	1	>0.9	1	0.9	**2**	**0.038**	1.14	0.3	1.18		1.5	0.5	0.21	0.2	1.9	0.077
iss[b]	0.34	0.085	0.9	0.6	0.8	0.3	0.9	0.5	1.2	0.3	0.7	0.3	**1.08**	**<0.001**	0.59		1.66	0.6	0.71	0.8	1	>0.9
sit.ep	1.58	0.5	0.6	0.058	0.6	0.08	1	>0.9	0.9	0.8	1.1	0.9	1.02	0.088	1.29		-	>0.9	-	>0.9	1.5	0.4
ompA	1.25	0.6	0.7	0.086	**0.6**	**0.01**	1.1	0.7	0.7	0.11	1.3	0.4	**1.6**	**0.018**	1.94		**2.39**	**0.004**	-	>0.9	**3.4**	**0.014**
iroN	0.52	0.13	0.9	0.6	0.6	0.11	1.1	0.7	1.1	0.9	1.3	0.6	**0.83**	**0.042**	1.38		0.6	>0.9	0.91	>0.9	1.4	0.5
sit.chr	0.44	0.3	0.9	0.6	0.6	0.071	0.7	0.2	0.9	0.8	0.4	0.11	1.51	0.14	0.87		1.27	0.3	1.42	0.8	0.7	0.5
traT[b]	0.35	0.2	1.2	0.4	1.2	0.5			1.3	0.3	1.8	0.11	**1.8**	**<0.001**	1.23		1.75	0.086	0	>0.9	1.2	0.7

Notes: OR: odds ratio; p: p-value; Bold p: Significant association ($p \leq 0.05$) was shown by binary logistic regression analysis; When $p \leq 0.05$, then OR > 1 means that VAG is introduced as predictor of resistance to the antibiotic agent, but OR < 1 means that VAG is a predictor of susceptibility to antibiotic agent.

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3.4 Associations between virulence genes harbored and antibiotic resistance genes in APEC isolates

Table 7: Univariable associations between virulence associated genes and antibiotic resistance genes among avian pathogenic *Escherichia coli* isolates from broiler and broiler breeder chickens (n = 331)

	Mcr1		*bla*$_{TEM}$		*Sul1*		*qnrA*		*tetB*		*Cat1*		*ereA*		*aac(3)-IV*	
	OR	**p**	**OR**	**p**	**OR**	**p**	**OR**	**p**	**OR**	**p**	**OR**	**p**	**OR**	**p**	**OR**	**p**
MalX	0.75	0.3	**3.04**	**<0.001**		>0.9	**7.28**	**<0.001**	**0.21**	**0.001**	0	>0.9		>0.9		>0.9
fimH		>0.9		>0.9		>0.9		>0.9		>0.9		>0.9		>0.9		>0.9
kpsIII	1.51	0.1	1.59	0.06	**0.27**	**0.003**	**2.05**	**0.015**	0.61	0.084	**1.79**	**0.04**	1.36	0.6	2.05	0.15
ireA	**0.48**	**<0.001**	0.81	0.3	**0.34**	**<0.001**	**3.65**	**<0.001**	1.01	>0.9	**0.53**	**0.007**	1.51	0.5	0.61	0.3
fyuA	**0.37**	**<0.001**	**0.58**	**0.019**	0.94	0.8	0.91	0.8	0.99	>0.9	0.79	0.4	0.79	0.7	2.29	0.068
ironEC	**0.42**	**0.001**	0.44	**0.001**	1.59	0.2	**3.76**	**0.012**	**2.58**	**0.004**	**0.54**	**0.031**		>0.9		>0.9
sfa.foc	**0.29**	**0.002**	0.66	0.3	**2.55**	**0.011**	**3.72**	**<0.001**	**0.2**	**0.009**	0.89	0.8	**3.76**	**0.05**	**16**	**<0.001**
aerJ	**0.54**	**0.003**	**0.35**	**<0.001**	**3.08**	**<0.001**	**0.15**	**<0.001**			**2.29**	**0.005**	0.56	0.3		>0.9
papGIII	0.91	0.7	1.55	0.074	0	>0.9	**9.81**	**<0.001**	0	>0.9	0	>0.9	**3.86**	**0.015**		>0.9
ompT	**1.68**	**0.009**	**3.18**	**<0.001**	**0.23**	**<0.001**	**5.98**	**<0.001**	**0.12**	**<0.001**	1.26	0.4		>0.9	0.86	0.7
papGI	**0.31**	**<0.001**	**0.26**	**<0.001**	1.39	0.14	1.27	0.3	**3.51**	**<0.001**	**0.23**	**<0.001**	1.11	0.9		>0.9
kpsII	1	>0.9	**0.59**	**0.017**	0.62	0.061	**0.41**	**<0.001**	1.34	0.2	1.02	>0.9		>0.9		>0.9
papC[a]	**0.58**	**0.003**	**0.32**	**<0.001**	1.33	0.2	**0.26**	**<0.001**	**5.84**	**<0.001**	**0.56**	**0.013**	3.27	0.072		>0.9
cvaC	1.15	0.4	0.87	0.5	**4.56**	**<0.001**	**0.3**	**<0.001**	1.02	>0.9	0.71	0.2		>0.9	0.76	0.6
traT[a]	**0.39**	**0.005**	0.98	>0.9	1.62	0.3		>0.9	1	>0.9	0.62	0.2	**0.19**	**0.004**	**0.34**	**0.045**
papGII	1.03	>0.9		>0.9	**3.35**	**0.002**		>0.9	**7.39**	**<0.001**		>0.9		>0.9		>0.9
papGII.III	0.71	0.3	0.5	0.092	1.26	0.6	0.63	0.4	**2.01**	**0.042**		>0.9		>0.9	**4.1**	**0.009**
iha	1.57	0.15	**3.79**	**<0.001**	**1.95**	**0.046**		>0.9	0.96	0.9	**3.34**	**<0.001**	**5.56**	**0.003**	0	>0.9
iss[a]	0.61	0.15	0.69	0.3			**0.4**	**0.012**	**3.33**	**0.014**	1.13	0.8		>0.9		>0.9
etsB	**0.61**	**0.01**	**0.57**	**0.005**	1.23	0.4	**0.39**	**<0.001**	**4.43**	**<0.001**	1.14	0.6	0.72	0.6	1.79	0.3
colM	1.33	0.2	1.01	>0.9	0.72	0.2	**3.86**	**<0.001**	**0.34**	**<0.001**	**0.39**	**0.003**	1.92	0.2	0.77	0.6
colB	**0.67**	**0.034**	**0.65**	**0.027**	1.58	0.059	**0.38**	**<0.001**	**5.8**	**<0.001**	1.45	0.13	0.89	0.8	2.2	0.13
eitB	**0.61**	**0.008**	**0.47**	**<0.001**	1.14	0.6	**2.67**	**<0.001**	0.99	>0.9	**0.26**	**<0.001**		>0.9	0.62	0.3
eitA	1.13	0.5	0.84	0.4	0.9	0.6	**3.06**	**<0.001**	**2.18**	**<0.001**	**0.35**	**<0.001**		>0.9	0.9	0.8
tsh	0.81	0.3	0.98	>0.9	**2.21**	**<0.001**	**1.67**	**0.046**	0.88	0.5	0.96	0.9		>0.9	**5.94**	**<0.001**
iucD	0.72	0.11	**0.47**	**<0.001**	0.78	0.3	0.89	0.7	0.94	0.8	1.32	0.3	**0.21**	**0.006**	2.47	0.2
cvi.cva	**0.63**	**0.024**	1.09	0.7	**1.87**	**0.028**	0.66	0.12	1.1	0.7	0.81	0.4	1.39	0.6	0.94	0.9
irp2	0.88	0.5	1.23	0.3	0.77	0.3	**2.23**	**0.002**	1.26	0.2	0.86	0.5		>0.9	0.46	0.11
iss[b]	0.89	0.6	**0.47**	**<0.001**	**0.25**	**<0.001**	1.23	0.5	0.81	0.3	0.74	0.2	0.62	0.4	**0.36**	**0.025**
sit.ep	**0.6**	**0.037**	0.62	0.088	**0.46**	**0.038**	0	>0.9	**3.11**	**<0.001**	0.83	0.6		>0.9	0.86	0.8
ompA	**1.76**	**0.004**	**1.6**	**0.018**	**0.45**	**0.003**	0.66	0.13	**2.52**	**<0.001**	1.18	0.5	1.07	>0.9	0.76	0.6
iroN	0.98	>0.9	**0.58**	**0.042**	**2.11**	**0.005**	**2.1**	**0.01**	**0.06**	**<0.001**	1.02	>0.9		>0.9	**4.39**	**0.001**
sit.chr	1.33	0.3	1.53	0.14	**0.17**	**<0.001**	2.23	0.072	1.07	0.8	0.79	0.4	0.64	0.5	**0.09**	**<0.001**
traT[b]	0.66	0.086	**0.26**	**<0.001**	0.81	0.5	**0.53**	**0.032**	0.69	0.13	**0.49**	**0.009**	0.79	0.7	0.53	0.2

Notes: OR odds ratio, p p-value; Bold p: Significant association ($p \leq 0.05$) was shown by binary logistic regression analysis; When $p \leq 0.05$, then OR > 1 means that virulence associated gene is introduced as predictor of antibiotic resistance gene, but OR < 1 means that virulence associated gene is a predictor of another antibiotic resistance gene

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The malX, *kpsIII*, *ireA*, *ironEC*, *sfa.foc*, *papGIII*, *ompT*, *colM*, *eitB*, *eitA*, *irp2* genes were significantly more prevalent in isolates positive for *qnrA* genes in comparison to other resistant genes. Similarly, the *papGI*, *papC*^{a}, *papGII*, *iss*^{a}, *etsB*, *colB*, *sit.ep.,* and *ompA* genes were significantly more prevalent in isolates positive for *tetB* genes in comparison to other resistant genes. The *aac(3)-IV* ARG was associated with the presence of *aerJ* (OR = 16, p < 0.001) and *papGII.III* (OR = 4.1, p < 0.009). The *ereA* ARG was associated with the presence of *iha* (OR = 5.56, p < 0.003). The *sul1* ARG was associated with the presence of *aerJ* (OR = 3.08, p < 0.001), and *cvaC* (OR = 4.56, p < 0.001), *tsh* (OR = 2.21, p < 0.001), and *cvi.cva* (OR = 1.87, p < 0.028). A positive as well as negative association was observed between the *mcr1*, *blaTEM*, and *cat1* genes depending on the VAGs.

3.5 Associations between phenotypic antimicrobial resistance, and antibiotic resistance genes harbored in APEC isolates

The *tetB* gene was significantly more prevalent in isolates showing phenotypic resistance to tetracycline (OR =1.91, p= 0.03), chloramphenicol (OR =1.83, p = 0.003) and neomycin (OR =3.61, p = 0.039) (Table 8). The occurrence of *bla*$_{TEM}$ gene was significantly less prevalent in isolates with phenotypic resistance to tetracycline (OR =0.61, p = 0.048). Likewise, the occurrence of *aac(3)-IV* gene was significantly less prevalent in isolates with phenotypic resistance to tetracycline (OR =0.3, p = 0.01) (Table 8).

Table 8: Binary logistic regression analysis of antibiotic resistant genes as predictor of resistance to various antibiotic agents

Characteristic	*Mcr1*		*bla*$_{TEM}$		*Sul1*		*qnrA*		*tetB*		*Cat1*		*ereA*		*aac(3)-IV*	
	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p
Neomycin (NE, 10µg)	0.88	0.7	1.83	0.2	6.95	0.059	4.98	0.12	**3.61**	**0.039**	0.52	0.14	0.33	0.2	1.18	0.9
Gentamicin (GEN, 10µg)	0.85	0.4	0.91	0.6	0.94	0.8	0.9	0.7	1.06	0.8	0.86	0.5	1.33	0.6	0.89	0.8
Amikacin (AK, 30)	0.85	0.4	0.95	0.8	1.28	0.3	1.06	0.8	0.98	>0.9	0.78	0.4	0.47	0.3	1.47	0.4
Ceftriaxone (CRO, 30 µg)	1.03	0.9	1.05	0.8	0.81	0.4	1.31	0.3	1.01	>0.9	1	>0.9	1.66	0.4	1.67	0.3
Trimethoprim/ Sulphamethoxazole (TS 250µg)	1.06	0.8	0.82	0.3	1.3	0.3	1.55	0.12	0.97	0.9	1.01	>0.9	0.36	0.062	0.99	>0.9
Doxycycline (DXT, 30µg)	1.07	0.8	0.74	0.3	0.76	0.4	0.83	0.6	0.84	0.6	1.93	0.2		>0.9	0.41	0.12
Tetracycline (T30 µg)	0.98	>0.9	**0.61**	**0.048**	0.79	0.4	0.85	0.6	**1.91**	**0.03**	1.77	0.11	1.2	0.8	**0.3**	**0.01**
Chloramphenicol (C, 30µg)	1.19	0.3	1.27	0.2	0.66	0.068	1.15	0.6	**1.83**	**0.003**	0.82	0.4	0.77	0.6	0.5	0.15
Ciprofloxacin (CIP, 5µg)	1.34	0.6	0.41	0.2	0.66	0.6	0.48	0.3	1.15	0.8	1.03	>0.9		>0.9		>0.9
Enrofloxacin (ENF, 10 µg)	1.67	0.6	0.76	0.8	0.37	0.3	0.27	0.2		>0.9	0.92	>0.9		>0.9		>0.9
Levofloxacin (LEV, 5µg)	1.52	0.2	1.12	0.8	0.71	0.4	2.02	0.3	0.81	0.6	1.12	0.8		>0.9	0.72	0.7

Notes: OR: odds ratio, p: p-value; Bold p: Significant association (p ≤ 0.05) was shown by binary logistic regression analysis; When p ≤ 0.05, then OR > 1 means that antibiotic resistance gene is introduced as predictor of that phenotypic antibiotic resistance, but OR < 1 means that antibiotic resistance gene is a predictor of other phenotypic antibiotic resistance

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DISCUSSION

Colibacillosis is a significant poultry disease caused by avian pathogenic *E. coli*. Unlike most enteric pathogenic *E. coli*, extraintestinal infections associated with APEC are rarely linked with a specific set of established AMR and virulence-related factors. The rise of AMR in animals used for food production poses a substantial threat to public health and complicates veterinary prevention and treatment strategies. Transferable plasmids and chromosomes, including transposons, integrons, and genetic cassettes, harbor genetic factors contributing to AMR and virulence. Plasmids are crucial in HGT in bacterial populations, especially in Enterobacterales [24].

This study revealed diverse phenotypic and genotypic resistance patterns to commonly prescribed antibiotics in commercial chickens. Prudent therapeutic antibiotic use may aid in preventing or controlling colibacillosis on chicken farms. However, challenges arise from the emergence of MDR-APEC strains and the spread of resistance genes [25]. Earlier, our recent study [21] provided evidence that the poultry farms could indeed be contaminated with MDR-APEC with diverse phylogenetic groups among the different type of commercial chickens, especially with the potentially pathogenic B1, E, and A phylogenetic types [20, 21]. Our findings suggested that the isolates belonging to phylogenetic groups C, D and E clade were more susceptible to various antimicrobials than the isolates of other phylogenetic groups. **The increased susceptibility of phylogenetic groups C, D and E clade to various antimicrobials is due to fewer resistance gene, less frequent gene transfer event and different evolutionary pressures in these phylogenetic group.**

Furthermore, APEC strains mostly belonged to group B1, which showed the strongest pattern of antimicrobial resistance [20, 21]. The phylogenetic group B1 had significant association with neomycin, ceftriaxone, chloramphenicol, cotrimoxazole and doxycycline which is in agreement with Saha, Hoque (26). However, the findings from this study demonstrated that strains belonging to phylogenetic group B1 were found to carry more resistance properties and phylogenetic group D contained more VAGs, corroborating the previous finding [26-28]. It was also reported that isolates belonging to phylogenetic group B2 [29] and A1 [26] were more sensitive to drugs, despite their higher virulence. Similar to study reported by Saha, Hoque (26), we demonstrated that APEC isolates from all phylogenetic groups possessed MDR properties, which did not comply with the findings of Etebarzadeh, Oshaghi (30) and [27], who reported that only the phylogenetic group B2 could harbor isolates bearing MDR properties. The occurrence of phylogenetic group B1 with high AMR could be attributed to genetic transformation during multiplication. Horizontal transfer of resistance genes between APEC strains via plasmids might contribute to variations. The significant association of AMR with the prevalent genetic backbone (phylogenetic group) indicates more challenges in treating infections in the future, particularly concerning the WHO's critical importance antimicrobials [31]. This is particularly alarming in context of Nepal because of high disease burdens, emergence of resistance traits, and the confluence of prevailing socio-economic, demographic, and

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environmental factors. This study, the first to report the association of MDR-APEC phylogenetic groups in avian colibacillosis in Nepal, implies that poultry products in the country might act as a reservoir for resistant strains. Unfortunately, no APEC isolate showed sensitivity to all of the 14 antibiotics tested, potentially due to the widespread, indiscriminate antibiotic use and environmental contamination in poultry farms [32-35].

Furthermore, in the present study, isolates from different phylogenetic groups showed the same resistance patterns (R-types) and vice versa that indicates phenotyping might not be a robust genotyping strategy to predict AMR [36] except for few antimicrobials. Bacteria from different phylogenetic groups had the same resistance patterns due to the horizontal transfer of resistance genes, often carried on plasmids, which are not accounted for in phenotypic classifications based on chromosomal genes. It is not surprising due to the fact that general bacterial classification in Clermont's method is based on some conserved chromosomal genes (like *chuA*, *yjaA*, and the DNA fragment *TspE4.C2*). However, this method does not consider plasmid-borne genes, which play a pivotal role in antibiotic resistance. As a result, bacteria classified into different phylogenetic groups by Clermont's method can still exhibit similar resistance profiles if they harbor similar plasmids carrying resistance genes. Furthermore, plasmids which have a pivotal role in AMR are not relevant in Clermont's method. Furthermore, plasmids are extrachromosomal DNA elements that can move between bacteria through horizontal gene transfer, spreading resistance genes across different species and strains. This mechanism significantly complicates the prediction of resistance patterns based solely on chromosomal gene analysis [37, 38].

Regarding the relationship between AMR and VAGs, two hypotheses have been proposed. Initially, the coexistence of VAGs and AMR genes within various genetic elements, such as integrons, composite transposons, and plasmids, suggests that strains resistant to antimicrobials may exhibit increased virulence. Nevertheless, considering that virulence and AMR do not always emerge simultaneously, some researchers suggested strains with AMR might be less virulent compared to susceptible ones [16, 39-44]. In the present study, ciprofloxacin and tetracycline resistant isolates had a significantly higher number of virulence genes [42, 45]. These hypotheses highlight the complex interaction between AMR and virulence, where the presence of both types of genes on MGEs can lead to the evolution of more dangerous strains, yet not all antimicrobial-resistant strains exhibit enhanced virulence. Interestingly, the top two most prevalent VAGs had no association with corresponding phenotypic resistance. In contrary to our finding, *ompT* was the most prevalent gene in ciprofloxacin and tetracycline resistant isolates [41]. In addition, the occurrence of *ironEC*, *traT*^{b}, *kpsII* and *iss*^{b} in tetracycline resistant isolates was significantly higher than the susceptible genes. The occurrence of *ironEC*, *kpsII* in gentamicin sensitive and cvi*/cva* in amikacin was significantly higher in resistant isolates than other. In our investigation, we identified several significant associations between virulence and antimicrobial resistance at the isolate level. Notably, positive correlations were observed between the presence of *ompA* with levofloxacin, ciprofloxacin and tetracycline resistance as well as between *irp2* and resistance to gentamicin and

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doxycycline. Additionally, the presence of *fyuA*, *ironEC*, *aerJ*, *papGI* and *papC* were found to be linked to the significant increase in the number of antimicrobials to which an isolate exhibited resistance. The concerning aspect lies in the associations between resistance to multiple antimicrobials and the presence of several virulence genes considered important for APEC virulence. This is alarming as infections caused by virulent, multidrug resistant strains of *E. coli* could lead to substantial economic losses in broiler flocks, owing to increased morbidity, mortality, and a limited array of effective antimicrobial treatment options. Given the potential for resistance to develop even in the absence of direct antimicrobial selection pressure through co-selection [46] or HGT [17-19], future research should prioritize seqencing and plasmid profiling as it would help to determine where some of virulence or resistance genes are located. Such investigations are crucial for devising strategies to mitigate the impact of these pathogens on public health and agriculture systems.

The logistic regression analysis revealed that *colM* gene, *ompA* gene and *tsh* gene could be a predictor for neomycin resistance, levofloxacin resistance and amikacin resistance, respectively. In some different studies, *papC* gene was identified as a predictor for ampicillin resistance [47, 48]. Contrary to the results reported by Yazdanpour, Tadjrobehkar (48), we also observed significantly reduced occurrence of virulence genes, including *malX*, *kpsIII*, *cvaC*, *iha*, *iucD*, *iroN* and *sfa.foc* in antibiotic-resistant isolates compared to antibiotic-susceptible isolates. Furthermore, Yazdanpour, Tadjrobehkar (48) reported *hlyA ompA, malX* genes to be predominant in resistant isolates. However, in this study, we found that *ompA*, *colM, papGII/III* and *kpsII* genes were obviously more abundant in the isolates resistant to different antibiotic agents. Moreover, isolates containing all these genes (*fyuA*, *ironEC*, *aerJ*, *ompT*, *kpsII*, *papGI*, *papC*, *colB*, *colM*, *traT*, *iss*, *etsB*, *irp2*, *tsh*, *cvi.cva*, *eitB*, *papGII.III* and *ompA*) were significantly more prevalent in resistant isolates, particularly in relation to ciprofloxacin and tetracycline resistance. Consequently, *ompA* and *papC* genes are suggested as possible predictors of antibiotic resistance in MDR-APEC isolates [18, 49].

Similarly, there was a significant presence of virulence genes associated with antibiotic resistance genes for fluroquinolones and tetracycline (*qnrA* and *tetB*). The specific host, geographical origin, and type of antimicrobial agent used may potentially influence the relationship between AMR and VAGs [50]. Additionally, the genotypic resistance in bacteria may involve transposons and integrons besides plasmids. A diverse array of VAGs were frequently identified in APEC isolates, potentially contributing to their pathogenicity in avian colibacillosis. These genes include those involved in the synthesis of invasins, toxins, siderophores, adhesions, and iron transport mechanisms. Several VAGs, including *fimH*, *iss*^{a}, *traT*^{a}, *sit chr.*, *ironEC*, *kpsII*, *iss*^{a}, *cvi/cva*, *iucD*, *aerJ* and *ompT* play a crucial role in APEC adherence. VAGs tested in this study were detected in 99.2 % of APEC isolates. Likewise, 90.1% of strains had at least one ARG and 81.9% are assigned to a phylogenetic group. The remaining isolates might harbor different set of genes that were not included in the multiplex PCR.

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The top four VAGs, including adhesin *fimH*, *traT*[a], *sit chr* and iron uptake *iss*, identified as minimal predictors, are highly conserved and widespread among APEC strains [26, 51]. This suggests evidence for them being marker gene of APEC. Additionally, the association of *iss*[b] with different phylogenetic groups was reported by Rodriguez-Siek, Giddings (52). The high prevalence of iron uptake associated gene in our study may be attributed to the universal presence of this genes among the APEC [18]. The occurrence of the other three genes of the APEC pentaplex [17], namely the outer membrane protein *ompT* and *iucD*, and the membrane siderophore receptor *iroN*, was highly associated with phylogenetic group D and E. clade respectively in our study. The current study underscores the significance of phylogenetic group D strains in the development of colibacillosis, as evidenced by the virulence factors *fimH*, *iroN*, *iss*, *traT*, *ompT*, *iucD*, *tsh, etsA*, *etsB*, fyuA, *irp2*, *cvaC,* and *cvi.cva,* which are located extrachromosomally on pathogenicity islands (ColV or ColBM virulence plasmids). The phylogenetic group D was the most virulent and are most resistant group in another study too [38]. APECs phylogenetic groups association with VAGs and AMR suggests phylogroup D is very important for colibacillosis.

Phylogenetic group analysis showed that the majority of APEC isolates belonged to phylogroup B1 (19.7%), consistent with findings in other studies [53, 54]. Notably, broilers, the primary population studied, were predominantly led by Group E (26.7%), supported by Munkhdelger, Gunregjav (55). Most VFs exhibited significant correlations with phylogenetic group D and E in our study, followed by phylogenetic group C, and E. clade. However, *sfa.foc* genes were more prevalent in phylogroup B2, indicating their potential as a predictor of Group B2 origin, and their significant association with phylogroup B2 with APEC, as supported by Munkhdelger, Gunregjav (55) in UPEC.

Contrary to previous studies, we observed phylogenetic group E and B1 as most prevalent in large populations of broiler and broiler breeder, whereas B2 and D (pathogenic strains) were less predominant [6, 54, 56-61]. While phylogenetic group A and B1 are often considered commensal [62], strains belonging to phylogenetic group B2 and D phylogroups are known for causing majority of infections caused by ExPEC [63]. Although phylogenetic group B1 is predominant in our study, with significant association of VAGs like *iroN*, *ompA*, *colB*, *etsB* and *aerJ,* the occurrence was lower compared to other phylogenetic groups [64].

Intriguingly, our results contradict the common assertion that phylogenetic groups D and E are the most virulent and carry the highest number of VAGs. The acquisition of VAGs by commensal groups, such as phylogenetic group A, is possible, as suggested by previous studies [62]. According to Dziva and Stevens (65), commensal *E. coli* in the intestine can transform into APEC, acquiring virulence genes like *fimH* (Type 1 fimbriae), *tsh* (Temperature-sensitive hemagglutinin), *iss* (Increased serum survival), and iron acquisition system genes. Our investigated strains possess genes for iron-scavenging systems (*iss* and *fimH*), suggesting their potential to transition from commensal to pathogenic.

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Of the 34 VAGs analyzed, 11 were significantly associated with phylogenetic group D, indicating a substantial potential for hosting plasmid-mediated VAGs and ARGs [66], disseminating among bacterial hosts [24]. This aligns with findings by Tohmaz, Askari Badouei (66) regarding the significant presence of VAG with phylogenetic group C, D. In contrast to our findings, previous studies identified the most frequent genes, namely *fimH*, *iss*, *kpsII*, and *ompT*, with phylogenetic groups other than B2 [26]. Our study identified *papC*, *tsh*, *irp2* and *cvaC* as predictor of phylogenetic group C. Another study identified *irp2* as a predictor of phylogenetic group A [47, 48]. The statistical association observed between VAGs and phylogenetic groups indicates the distinct VAG profiles associated with individual phylogenetic groups, highlighting the genomic diversity among *E. coli* strains causing urinary tract infections and avian colibacillosis.

While the Clermont PCR technique identified strains as phylogenetic group D, a more robust approach for phylogenetic group identification is multi-locus sequencing typing (MLST), as suggested by Dissanayake, Octavia (61). Strains identified through PCR may require further confirmation by MLST, which is based on sequences analysis of housekeeping genes so this which Clermont also uses but MLST is more refined. Phylogenetic grouping, which relies on identifying chromosomal markers, may not effectively account for virulence factors carried on plasmid-mediated pathogenicity islands (PAIs) and other extrachromosomal and MGEs. These extra-chromosomally located PAIs (ColV or ColBM virulence plasmids) (*iroN*, *iss*, *iucD*, *tsh* and *cvi.cva*) are considered crucial for APEC virulence causing avian colibacillosis [67]. Its association with ARG or phylogenetic group or AMR suggests that these genes might be co-located on plasmids and transmitted by HGT. Thus, the virulence of *E. coli* in birds may not be reliably predicted by PCR-based phylogenetic typing. The research underscores the challenge of defining APEC or human ExPEC isolates in absolute terms [12, 68, 69]. There is overlap among all ExPEC subgroups in terms of virulence genotypes, phylogenetic groups, and serogroups [12, 52]. Nevertheless, given that the majority of APEC isolates belong to phylogenetic groups other than the B2 group and carry the ColV or ColBM virulence plasmid, it can be inferred that these plasmids, along with certain other genetic elements shared by non-B2 types of avian *E. coli*, confer an increased capacity for avian colibacillosis on these strains.

This study investigates association of AMR with VAGs and phylogenetic groups in APEC from poultry farms in Nepal. The findings, while region-specific, may not represent APEC strains globally due to differing environmental and agricultural practices. Potential biases stem from limited sample size, limited VAGs and ARGs and selection methods, which might not capture the full diversity of APEC strains. Employing both phenotypic and genotypic methods revealed discrepancies between resistance patterns due to HGT. The Clermont PCR method, used for phylogenetic typing, relies on chromosomal markers and does not account for plasmid-borne genes crucial for understanding AMR and virulence. MLST could offer higher resolution. The study acknowledges HGT's role in spreading resistance and virulence genes but does not explore these mechanisms comprehensively.

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Plasmids, which are pivotal in AMR and virulence, were not extensively profiled for diversity and distribution within bacterial populations. Environmental contamination and indiscriminate antibiotic use were noted but not quantified. Variability in farm management practices, such as biosecurity measures and sanitation standards, can significantly influence AMR and virulence traits but were not systematically examined. While the study identified associations between certain VAGs and AMR genes, it did not delve deeply into the molecular mechanisms underlying these interactions. The hypotheses regarding VAGs and AMR gene co-existence were not conclusively tested, necessitating more targeted experimental studies. The study also discussed potential public health implications but lacked concrete data on MDR-APEC transmission from poultry to humans and did not quantify the economic impact of colibacillosis and AMR in poultry farming. Future research should integrate data from multiple sources, such as sample from defined clinical findings (colibacillosis, egg peritonitis and omphalitis etc), environmental samples and human clinical isolates, and include comprehensive genomic analyses to provide a holistic view of the AMR and virulence landscape. More specific recommendations for longitudinal studies are needed to track the evolution of AMR and VAGs in APEC over time.

CONCLUSION

The current investigation revealed significant correlations among the phenotype of APEC stains, virulence genotype, AMR genotype, and phylogenetic group. This study underscores the importance of exploring the interconnections between virulence genotype, genetic background, and AMR in APEC strains. Understanding these relationships is crucial for addressing the challenges associated with virulence-associated genes, antimicrobial resistance, and their impact on poultry production, as well as food safety and public health. Despite these findings, pinpointing the precise variables influencing the relationship between phylogenetic grouping and pathogenicity in APEC remains challenging. Unravelling the reasons behind the greater propensity of specific phylogenetic groups to acquire and express virulence features is essential for predicting the emergence of highly pathogenic APEC strains. Given the complexity of APEC infections, a comprehensive understanding of the phenomenon is vital for effective management and control.

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