



UNIVERSITI PUTRA MALAYSIA

**OCCURRENCE OF AVIAN PATHOGENIC *Escherichia coli* (APEC) IN
COMMERCIAL BROILER CHICKEN WITH CHRONIC COMPLICATED
RESPIRATORY DISEASE (CCRD)**

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FPV 2023 29**

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NUR AIMI SYIFA BINTI HUZAIRI

A project paper submitted to the
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In partial fulfillment of the requirement for the
DEGREE OF DOCTOR OF VETERINARY MEDICINE

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Identification of Avian Pathogenic Escherichia coli (APEC) in Commercial Broiler Chicken with Complicated Chronic Respiratory Disease (CCRD)”, by Nur Aimi Syifa binti Huzairi and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4901 – Project

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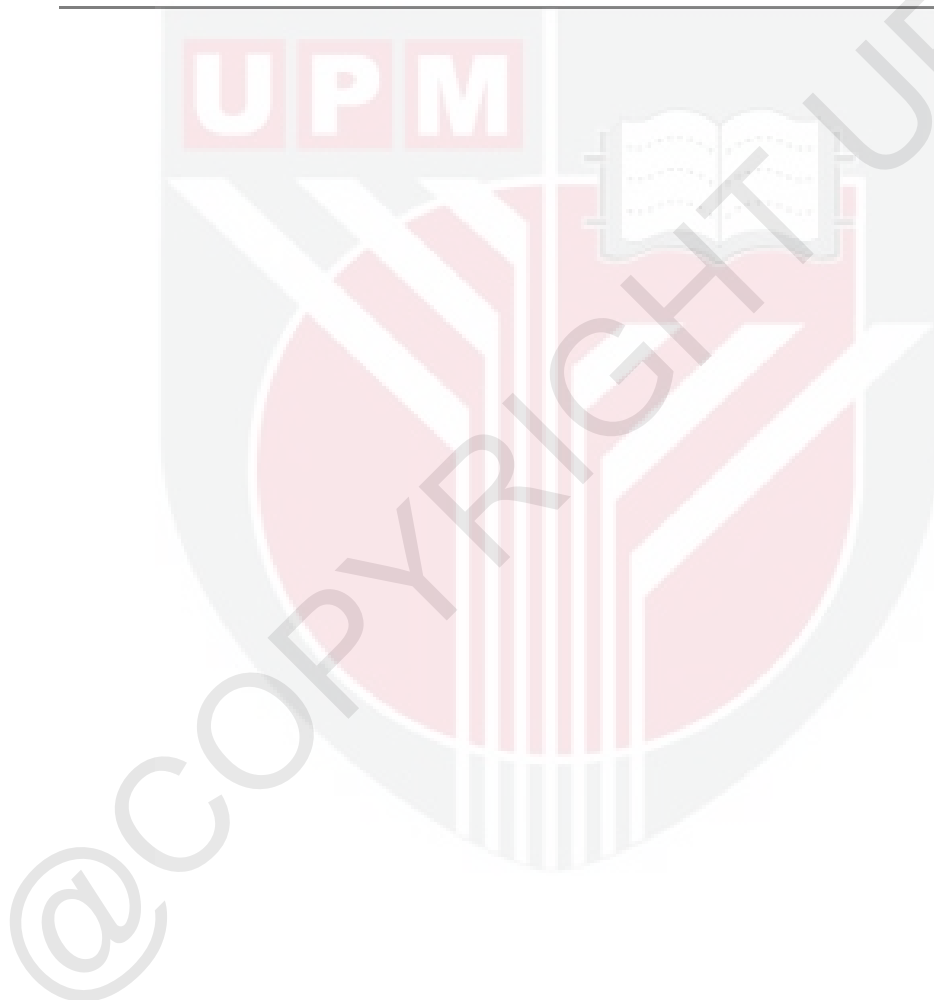
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LIST OF ABBREVIATIONS

Abbreviations	Caption
°	Degree
°C	Degree celcius
%	Percent
µm	Micrometer
µl	Microliter
ml	Milliliter
kg	Kilogram
bp	Base pair
rpm	Revolutions per minute
APEC	Avian Pathogenic <i>Escherichia coli</i>
AST	Antimicrobial sensitivity test
CCRD	Complicated Chronic Respiratory Disease
CLSI	Clinical and Laboratory Standard Institute
DNA	Deoxyribonucleic acid
DVS	Department of Veterinary Services
<i>E. coli</i>	<i>Escherichia coli</i>
H ₂ S	Hydrogen sulfide
LPS	Lipopolysaccharide

MDR	Multiple drug resistant
myGAP	Malaysian Good Animal Practices
PAIs	Pathogenicity islands
PCR	Polymerase Chain Reaction
SIM	Sulphide-Indole-Motility
TSI	Triple Sugar Iron
UV	Ultraviolet
VRI	Veterinary Research Institute
<i>astA</i>	Arginine succinyltransferase
<i>vat</i>	Vacuolating autotransporter toxin

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901 – Projek.

KEHADIRAN *Escherichia coli* PATOGENIK UNGGAS DI DALAM AYAM PEDAGING KOMERSIAL YANG MENGHADAPI PENYAKIT PERNAFASAN KRONIK KOMPLEKS

oleh

Nur Aimi Syifa binti Huzairi

2023

Penyelia: Profesor Dr. Zunita Zakaria

Kolibasilosis ayam biasanya dikaitkan dengan penyakit pernafasan kronik kompleks (CCRD) yang berpunca oleh *Escherichia coli* Patogenik Unggas (APEC). Ia dilaporkan sebagai penyakit bakteria utama dalam industri ayam yang menyebabkan kerugian ekonomi yang besar. Tujuan kajian ini adalah untuk menentukan kehadiran APEC pada ayam yang menunjukkan tanda-tanda CCRD seperti bengkak pada bahagian kepala, bulu yang tidak terurus dan pertubuhan yang terbantut. Sampel diambil menggunakan swab kloaka daripada 30 ekor ayam di sebuah ladang ayam pedaging di Selangor. Sampel diinokulasi ke dalam agar 'MacConkey' dan agar nutrient untuk mendapatkan kultur yang diduga sebagai *E. coli*. Kemudian, identifikasi *E. coli* terhadap bakteria yang telah dikultur menggunakan rangkaian ujian biokimia yang melibatkan ujian oksidasi, ujian besi gula tiga (TSI), ujian sulfida-indol-motiliti (SIM), ujian urea dan sitrat. Seterusnya, reaksi rantai polimer multipleks (PCR) digunakan untuk mengenal pasti APEC dari kultur *E. coli* yang diperoleh. Lapan set primer iaitu *astA*, *iss*, *irp2*, *papC*, *iucD*, *tsh*, *vat* dan *cva/cvi* telah digunakan untuk mengenal pasti kehadiran genetik virulen dalam isolate *E. coli*. Isolate yang mempunyai empat atau lebih genetik virulen dianggap sebagai

Escherichia coli Patogenik Unggas (APEC). Profil kepekaan antibiotik bagi isolate APEC telah dijalankan menggunakan teknik penyebaran cakera 'Kirby Bauer' yang standard. Keputusan reaksi rantaian polimer multipleks menunjukkan terdapat pelbagai penyebaran genetik virulen di kalangan isolate. Daripada 42 isolate *E. coli*, tiga belas (31%) telah dikenal pasti sebagai APEC. Genetik virulen yang paling banyak dikenal pasti adalah virulen *iucD* dimana kesemua isolate APEC mengandungi virulen ini. Genetik kedua yang paling kerap adalah *iss* dan *irp2*. Sebaliknya, *astA* menunjukkan genetik yang paling sukar dijumpai kerana hanya satu daripada 13 isolate APEC yang mempunyai genetik virulen ini. 13 isolate APEC yang telah dikenal pasti, didapati 100% rintangan terhadap ampisilin dan tetrasiklin. Ia juga menunjukkan 77% rintangan terhadap amoksisilin dan reaksi yang di pertengahan sebanyak 69% terhadap enrofloksasin. Bagi gentamisin dan kloramfenikol, isolate ini menunjukkan kerentanan sebanyak 85% dan 77%. Kesimpulannya, terdapat kehadiran APEC dalam ayam yang menunjukkan tanda-tanda penyakit pernafasan kronik kompleks dengan genetik virulen *iucD* sebagai genetik yang paling kerap. Isolate APEC yang diperoleh ini juga boleh dianggap sebagai bakteria yang tahan pelbagai ubat.

Kata kunci: Ayam, Penyakit Pernafasan Kronik Kompleks (CCRD), *Escherichia coli* Patogenik Unggas (APEC), profil kerentanan antibiotik, reaksi rantaian polimer multipleks.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4901 – Project.

OCCURRENCE OF AVIAN PATHOGENIC *Escherichia coli* (APEC) IN COMMERCIAL BROILER CHICKEN WITH COMPLICATED CHRONIC RESPIRATORY DISEASE

By

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2023

Supervisor: Professor Dr. Zunita Zakaria

Avian colibacillosis is commonly associated with complicated chronic respiratory disease (CCRD) in chickens caused by Avian Pathogenic *Escherichia coli* (APEC). It is reported as the major bacterial disease in the poultry industry that leads to a considerably large economic loss. The purpose of this study was to determine the occurrence of APEC from chickens that were showing signs of CCRD such as swollen head, ruffled feathers and stunted growth. A total of 30 cloacal swabs samples chickens were collected from a broiler farm in Selangor. Samples were initially inoculated onto MacConkey agar and nutrient agar to isolate presumptive *E. coli* cultures. Subsequently, the identification of *E. coli* was done using a series of biochemical test which include oxidase test, triple sugar iron test, sulphide-indole-motility (SIM) test, urea test and citrate test. Next, multiplex polymerase chain reaction (PCR) was used to identify APEC from the *E. coli* isolates obtained. A total of eight primer sets namely *astA*, *iss*, *irp2*, *papC*, *iucD*, *tsh*, *vat* and *cva/cvi* were used to determine the presence of virulent genes in the isolates. Isolates possessing four or more virulent genes are considered as APEC. The antibiotic susceptibility profiles for the APEC isolates were conducted using the standard Kirby Bauer disc diffusion

technique. The results of the multiplex polymerase chain reaction showed that there were various distribution of virulence genes amongst the isolates. Thirteen out of 42 *E. coli* (31%) isolates were identified as APEC. All APEC isolates contain the *iucD* virulent gene. The second highest prevalent gene detected were *iss* and *irp2*. In contrast, *astA* showed the least prevalent gene as it was only found in 1 out of 13 APEC isolates that was identified. APEC isolates were found to be 100% resistant towards ampicillin and tetracycline. It also showed 77% resistant towards amoxicillin and high intermediate of 69% for enrofloxacin. For gentamicin and chloramphenicol, the isolates showed susceptibility of 85% and 77% respectively. In conclusion, there were presence of APEC in chickens presenting CCRD signs with *iucD*, an iron acquisition system gene as it most prevalent gene. The APEC isolates can be considered as multidrug resistant bacteria.

Keywords: Poultry, Complicated Chronic Respiratory Disease (CCRD), Avian Pathogenic *Escherichia coli* (APEC), antibiotic sensitivity test, multiplex polymerase chain reaction.

1.0 INTRODUCTION

Malaysian poultry industry is big and self-sufficient which is able to meet 98.4% of the national demand for poultry meat. Malaysia's chicken per capita consumption is considered to be amongst the highest in the world at 52 kg/person/year (Syafuddin et al., 2023). According to Drewnowski et al. (2020), the anticipation is that this trend will continue to rise in the future, given the expansion of the Malaysian economy, which had led to a shift in dietary patterns towards an increased consumption of meat particularly poultry products. However, to reach this expectation there are many challenges faced by the farmers. One of the primary challenges in the industry is disease because an infected chicken would lead to low production meat. Primarily known as Avian colibacillosis is one of the most common diseases infecting chickens of the poultry industry.

According to Kathayat et al. (2021), avian colibacillosis stands out as the primary reason for both mortality (reaching up to 20%) and morbidity among broiler chickens. The study highlights substantial losses for farmers, manifesting as a 2% reduction in live weight and a 2.7% decline in feed conversion rate due to this condition. Moreover, an elevated rate of carcass condemnation (up to 43%) is also noted consequently. Colibacillosis often occurs in conjunction with other diseases like Infectious Bursal Disease (IBD), Infectious Bronchitis (IB) and Newcastle disease (ND) but it is most frequently associated with mycoplasma, leading to the development of complicated chronic respiratory disease (CCRD).

The etiological agent of avian colibacillosis is the gram-negative bacterium, *Escherichia coli* (*E. coli*). *Escherichia coli* is a non-spore-forming bacillus capable of thriving in both aerobic and anaerobic environments. While it normally exists as part of the chicken intestinal microflora, the strains causing avian colibacillosis are not the

typical commensal *E. coli*. These pathogenic variants are referred to as Avian Pathogenic *Escherichia coli* (APEC). Different from the commensal *E. coli*, APEC have the ability to employ various virulence and pathogenesis factors leading to disease. Numerous studies have identified several virulence genes associated with APEC. However, these virulence factors are seldom present in the isolates indicating that APEC strains constitute a heterogeneous group. An *E. coli* isolate can be classified as APEC only if it harbours at least four out of eight specified virulence genes which are *papC*, *iucD*, *irp2*, *tsh*, *vat*, *astA*, *iss* and *cva/cvi*.

Kathayat et al. (2021) had discussed various strategies have been employed to mitigate and manage APEC infections in the poultry sectors. These include enhanced hygiene practices, vaccination, implementation of biosecurity measures and addressing environmental stressors. Additionally, alternatives like probiotics, bacteriophages, innate immune stimulants and antimicrobial peptides have been assessed, aiming to establish new preventive and therapeutic interventions for the control of colibacillosis in chickens. Nevertheless, the effectiveness of these approaches has some restrictions, prompting a reliance on antimicrobial therapy as the predominant method to control APEC infections.

Numerous studies regarding prevalence of virulence factors of APEC and its antimicrobial susceptibility had been done in many countries but there is still lack of study regarding this disease in Malaysia. A review article about an overview of APEC (Kathayat et al., 2021) had listed a summary of antibiotic resistance and resistance genes from various countries including our neighboring country such as Thailand and Indonesia but Malaysia is not found in the list. Hence, this study is conducted to review further regarding the occurrence of APEC in commercial broiler chickens with CCRD in Malaysia and its antimicrobial susceptibility.

The objectives of this study were:

1. to isolate and identify *Escherichia coli* from commercial broiler chicken with Complicated Chronic Respiratory Disease (CCRD)
2. to identify Avian Pathogenic *Escherichia coli* (APEC) from the isolates obtained.
3. to determine the antibiotic resistance of APEC isolates towards common antibiotics used to treat Avian colibacillosis in Malaysia



2.0 LITERATURE REVIEW

2.1 Background

Avian colibacillosis is caused by Avian Pathogenic *Escherichia coli* and is a commonly known disease in the poultry industry worldwide. This disease is one of the primary cause of fatality (up to 20%), morbidity and increase of condemnation of carcasses of chickens at the slaughterhouse (Kathayat et al., 2021). Singh (2022) reported that colibacillosis often occurs concurrently with various disease especially Mycoplasmosis which leads to Complicated Chronic Respiratory Disease (CCRD). Due to the presence of these mixture of diseases, it makes early diagnosis challenging due to its complexity.

2.2 Aetiological Agent

Escherichia coli (*E. coli*) is a gram-negative, non-spore forming bacillus that is considered as a normal microflora inside chicken intestines and any other animals (Nolan, 2023). These rod-shaped bacteria are typically 1 – 3 μm x 0.4 – 0.7 μm in size with around 1 μm long and motile. Optimal temperature for *E. coli* to grow is 37°C overnight. Besides that, simple nutrients such as glucose as carbon and energy source, ammonium salts as nitrogen source and trace element are required for *E. coli* culturing. Hence, it is easy to grow this bacterium inside a laboratory setting only by using common medium such as MacConkey agar and nutrient agar. Morphology of *E. coli* on a MacConkey agar are described as dark pink isolates, dry with rounded dark pink area of precipitated bile salts due to its ability to ferment lactose. On a nutrient agar, *E. coli* will form colonies that are large, thick, greyish white, moist, and smooth (Basavaraju & Gunashree, 2023).

However, those that can cause diseases are not the normal microflora *E. coli*. According to Nolan (2023), most instances of CCRD in chickens is the result of *E. coli* strains that have obtained a collection virulence gene grouped within pathogenicity islands (PAIs) carried by plasmids. These plasmids containing PAIs are considered the distinctive characteristic of APEC pathotype. Kathayat et al. (2021) explained that APEC have the ability to utilize different virulence and pathogenesis factors to cause disease depending on the virulence factors that the bacteria acquired. According to Thomrongsuwannakij et al. (2020), *E. coli* strain can only be classified as APEC if it harbour at least four out of eight virulence gene. Virulence genes that are considered are those that were listed in Ewers et al., (2004), which are *papC*, *iucD*, *ipr2*, *tsh*, *vat*, *astA*, *iss* and *cva/cvi*.

2.3 Epidemiology

Avian colibacillosis involves a complex relationship between factors influencing the prevalence, transmission and virulence of APEC strains. Various studies have highlighted the global distribution of APEC, emphasizing regional variations in prevalence linked to diverse geographical and climatic conditions (Manges et al., 2015). This journal stated European and North American studies similar distributions of APEC strains but for Asian and African studies were varied. For transmission of this disease, horizontal transmission through contaminated feed, water, and direct bird-to-bird contact are identified. Populations of *E. coli* persist in the farm environment due to fecal contamination. Nolan (2023) stated the initial exposure of APEC may occur in the hatchery through contact with infected or contaminated eggs. Points of entry for the bacteria into birds can vary which may include respiratory tract, wounds, cloaca and compromised intestinal

mucosa. In addition, host susceptibility is also a factor where establishment of infection are influenced by age, stress and presence of concurrent diseases. Young and immunocompromised birds being more susceptible to APEC-induced colibacillosis (Kathayat et al., 2021).

2.4 Clinical signs

Clinical signs shown by chickens infected with APEC varies from one chicken to another. External examination that could be observed are swollen face, eye discharge, nasal discharge, and difficulty in breathing (Nolan, 2023). This article also reported that chickens with these signs are prone to distress. Hence, chickens can be observed to be anorexic, slow growth, and decrease reproductive rate up to 30%.

According to Kamaruzaman et al. (2021), upon postmortem gross lesions present in a chicken infected with APEC revealed to have fibrous deposits on the bird's air sacs indicating air sacculitis, an enlarged heart with fibrinous coating of its pericardial sac known as pericarditis, perihepatitis and pleuritis. This article further reported the histopathological findings of the organ samples of APEC infected chickens. Sinusoidal structure of hepatic cells was no longer present, suggesting degeneration and necrosis of liver. Besides that, there was also an expansion of alveolar spaces observed in the lung tissue indicating the presence of pulmonary emphysema. Tracheal tissue had also shown high density of inflammatory and blood cells that are consistent with tracheitis and congestion.

2.5 Treatment

In Malaysia, according to Rachel et al. (2020), common antibiotics used to treat avian colibacillosis are enrofloxacin, amoxicillin, ampicillin, chloramphenicol, gentamicin and tetracycline. However, the treatment of colibacillosis with antimicrobial agents poses challenges due to the prevalent multidrug resistance among APEC and the constraints on antimicrobial use in poultry imposed by regulations and public concerns (Nolan, 2023). Therefore, as elaborated in the same article, the industry places greater emphasis on preventing colibacillosis through effective management practices aimed at reducing bird exposure to APEC. This preventive approach also seeks to minimize the impact of stress and underlying infections that could heighten the susceptibility of birds to APEC infection. Additionally, various types of experimental and commercial vaccines have been employed in efforts to prevent colibacillosis, yielding mixed results in their effectiveness.

3.0 MATERIALS AND METHODS

3.1 Sampling

Approval for animal sampling was obtained from Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia with reference number UPM/IACUC/AUP-U017/2023. Sampling was done at a commercial broiler chicken farm located in Selangor. Sampling method used was convenient and purposive method where only chicken showed clinical signs of complicated chronic respiratory disease (CCRD) was selected. Examination of the chickens were carried out to identify those having swollen heads (Figure 1), ruffled feathers (Figure 2), and stunted growth (Figure 3). In total, 30 cloacal swabs were collected from chickens presenting CCRD signs and transported back to Bacteriology Laboratory, Faculty of Veterinary Medicine, UPM using an icebox.



Figure 1: CCRD clinical signs of swollen head in chicken



Figure 2: CCRD clinical sign of ruffled feathers observed in chickens



Figure 3: CCRD clinical sign of stunted growth of chickens

3.2 Isolation and Identification

All swabs were cultured onto MacConkey agar (Oxoid Ltd.) as primary culture and incubated overnight at 37°C. Isolates were then subcultured onto a nutrient agar (Oxoid Ltd.) and grown overnight at 37°C to achieve pure culture. Only colonies that showed presumptive *E. coli* morphology which is dark pink, dry and rounded with dark pink area of precipitated bile salts were selected and

subcultured. Then, the isolates were identified using gram a series of biochemical tests which include oxidase test, triple sugar iron (TSI) test, sulphide-indole-motility (SIM) test, citrate test and urea test.

According to Microbiology Laboratory Manual by Naveena & Joy (2014), an isolate identified as *E. coli* should show negative oxidase test. An oxidase test is conducted using tetra methyl-p-phenylenediamine dihydrochloride, a colourless redox reagent that will turn into compound indophenol blue as it oxidized by oxidase enzymes. For an *E. coli* isolate, it will show no colour changes. Next, TSI test was conducted to identify the bacterial ability to ferment glucose, sucrose and lactose. *E. coli* will show yellow slant, yellow butt and presence of gas that is interpreted as acid slant, acid butt with gas and no production of hydrogen sulfide (H₂S) as *E. coli* is able to ferment glucose and either sucrose, lactose or both. SIM test is a combination of test to identify the ability of bacteria to produce H₂S, to decompose amino acid tryptophan to indole and the bacterial motility. *E. coli* is expected to show no production of H₂S, development of cherry red colour of the Kovac's reagent and diffused growth which indicates motility. Lastly, *E. coli* is expected to show negative results for both urea and citrate test where no colour change is observed. Inoculation of *E. coli* on urea test will leads to medium remains to be yellow while for citrate test, medium remains as green.

3.3 Genotyping

3.3.1 DNA template preparation

Fresh *E. coli* cultures on nutrient agar at 37°C were used for the preparation of DNA template. Boiling method was carried out to obtain crude extract DNA of each *E. coli* isolates. Each culture was suspended with 100 µl distilled water in

1.5 ml microfuge tubes. The cell suspension was heated up to 96°C for 10 minutes in a dry bath and proceeded to centrifuge it at 14, 000 rpm for 5 minutes. Supernatant were then used as the DNA templates. Reference culture *E. coli* IMT 2470 (APEC) was used as the positive control. This strain contains all the virulence genes tested in this study.

3.3.2 Primer

Primers specific for virulence gene reported by Ewers et al. (2005) were employed in this study. The genes are *astA*, *iss*, *irp2*, *papC*, *iucD*, *tsh*, *vat* and *cva/cvi*. Primer sequences and expected PCR product size are presented in Table 1.

Table 1: PCR primers sequences and product sizes.

Gene	Primer Sequence (5' - 3')	Size (bp)
<i>astA</i>	TGCCATCAACACAGTATATCC TCAGGTCGCGAGTGACGGC	116
<i>iss</i>	ATCACATAGGATTCTGCCG CAGCGGAGTATAGATGCCA	309
<i>irp2</i>	AAGGATTCGCTGTTACCGGAC AACTCCTGATACAGGTGGC	413
<i>papC</i>	TGATATCACGCAGTCAGTAGC CCGGCCATATTCACATAA	501
<i>iucD</i>	ACAAAAGTTCTATCGCTTCC CCTGATCCAGATGATGCTC	714
<i>tsh</i>	ACTATTCTCTGCAGGAAGTC CTTCCGATGTTCTGAACGT	824
<i>vat</i>	TCCTGGGACATAATGGTCAG GTGTCAGAACGGAATTGT	981
<i>cva/cvi</i>	TGGTAGAATGTGCCAGAGCAAG GAGCTGTTTGTAGCGAAGCC	1181

3.3.3 Multiplex Polymerase Chain Reaction

Mixture of polymerase chain reaction (PCR) cocktail was calculated prior to preparing it. The 50 μ l cocktail mixture contains 25 μ l of 2x MyTaq Red Mastermix (Bioline), one μ l from each forward and reverse primer of *astA*, *iss*, *irp2*, *papC*, *iucD*, *tsh*, *vat* and *cva/cvi* and nine μ l of DNA extract that was obtained. Amplification process was done with cycling protocol of t^1 , 3 min at 94°C; t^2 , 30 sec at 94°C; t^3 , 30 sec at 58°C; t^4 , 3 min at 68°C (t^2 - t^4 , repeated 25 times); t^5 , 10 min at 72°C (Schouler et al., 2012).

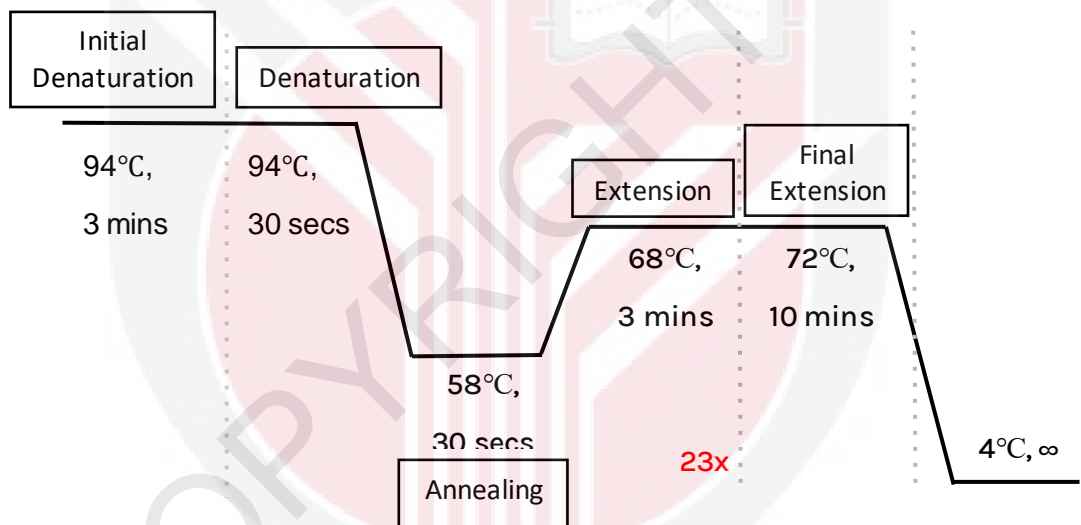


Figure 4: PCR protocol for APEC

3.3.4 Visualization of PCR products

Upon completion of the PCR reaction, the PCR products were visualized using 5 μ l of the reaction mixture in a 1.5% agarose, dissolve in 1x TBE (8.9 M Tris, 8.9M Boric Acid, 0.2M EDTA), for one hour at 90V. GelRed® Nucleic Acid Gel Stain was used for staining and results were captured under UV exposure.

3.4 Antibiotic Sensitivity Test

APEC positive colonies were transferred into a test tube containing 4 to 5 ml sterile saline. Turbidity of the mixture was adjusted with sterile saline to match the turbidity of 0.5 McFarland standard. A sterile swab was dipped and used to streaked it over the entire Mueller-Hinton agar surface. Streaking was repeated two more times by rotating the plate 90° each time to obtain an even distribution of the inoculum. Using a dispensing apparatus, antibiotic disc was placed evenly on the agar surface and was incubated for 16 – 18 hours at 37°C. Diameter of zone of inhibition were measured and interpreted to be either susceptible, intermediate, or resistant according to Clinical and Laboratory Standard Institute (CLSI) 3rd edition. Antibiotics that were used were Amoxicillin, Gentamicin, Enrofloxacin, Chloramphenicol, Tetracycline & Ampicillin. These antibiotics were chosen as they were listed in a Malaysian Good Animal Practices (myGAP) poultry farm survey conducted by DVS from year 2015 to 2017 as these are the most common antibiotics farmers used to treat avian colibacillosis.

3.5 Preservation of APEC cultures

APEC positive isolates were cultured into a slanted tryptic soy agar for preservation for future use.

4.0 RESULTS

All 30 plates of primary cultures MacConkey agar cultured from the cloacal swabs had presumptive *E. coli* colony morphologies. Few plates contained two types of presumptive *E. coli* colonies namely (A) shiny dark pink, dry and rounded with dark pink area of precipitated bile salt colonies and (B) non-shiny dark pink, dry and rounded with dark pink area of precipitated bile salt colonies. In total, there were 42 colonies of presumptive *E. coli* obtained. These cultures were purified on nutrient agar.



Figure 5: Shiny (Arrow) and Non-shiny (Arrowhead) colonies of presumptive *E. coli* on a MacConkey agar

All 42 cultures presented negative results for oxidase test. In addition, the isolates also showed similar reactions for TSI, SIM, urea and citrate test as shown in Figures 6, 7, and 8.

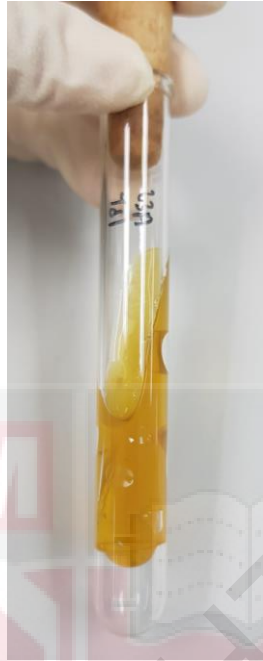


Figure 6: Acidic slant with acidic butt and gas produced on a TSI agar



Figure 7: Positive indole and motility in SIM test



Figure 8: Negative results were observed for urea and citrate test

Thus, all isolates were confirmed as *E. coli* isolates and subsequently show that the occurrence of *E. coli* in this broiler chicken farm is 100%.

Multiplex PCR genotyping of *E. coli* isolated from the cloacal swabs showed different combination of virulence gene from each sample (Figure 9, 10, 11 and 12)

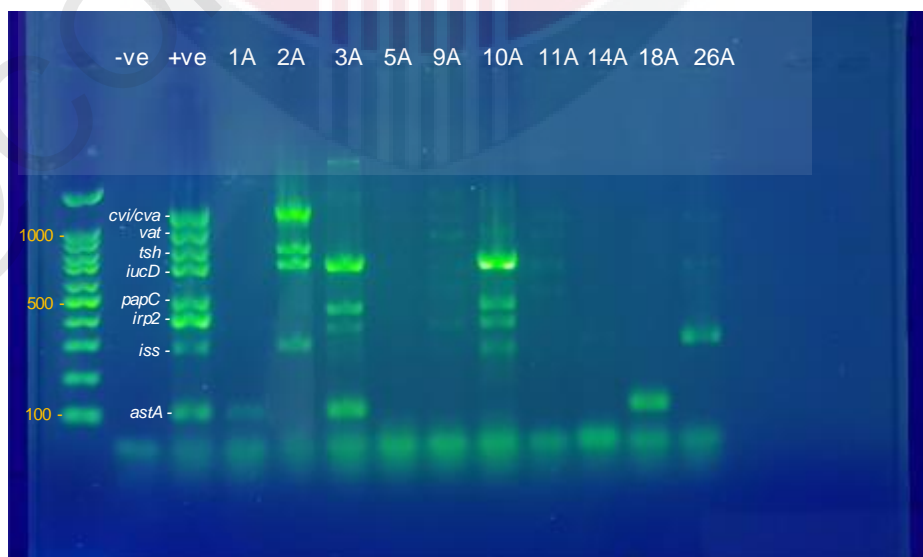


Figure 9: Multiplex PCR result (Batch 1)



Figure 10: Multiplex PCR result (Batch 2)



Figure 11: Multiplex PCR result (Batch 3)



Figure 12: Multiplex PCR result (Batch 4)

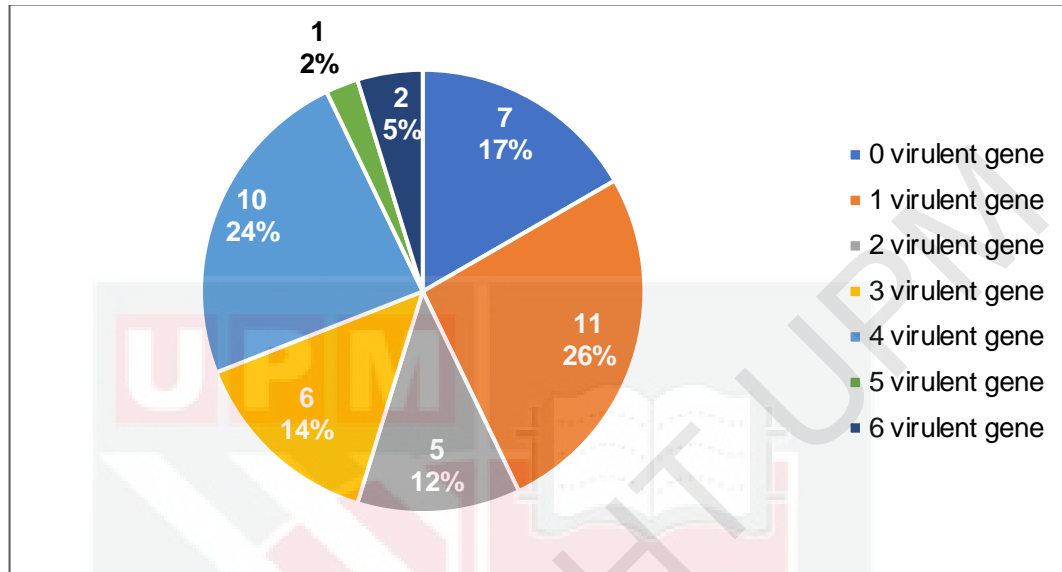
There were variations in distribution pattern of virulence genes between isolates as summarized in Table 2.

Table 2: Virulence gene distribution in all *E. coli* isolates

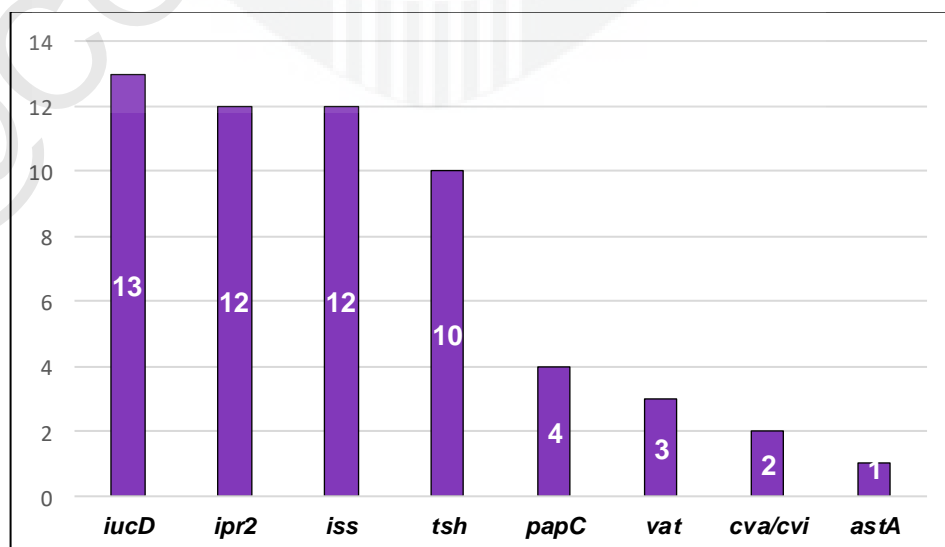
Sample No.	Presence of virulence-associated genes in <i>E. coli</i>								APEC
	<i>astA</i>	<i>iss</i>	<i>irp2</i>	<i>papC</i>	<i>iucD</i>	<i>tsh</i>	<i>vat</i>	<i>cva/cvi</i>	
IMT 2470	+	+	+	+	+	+	+	+	
1A									
2A		+			+	+		+	APEC 1
3A	+		+	+	+				APEC 2
4A									
5A									
6A	+								
6B	+	+							
7A	+	+			+				
7B			+						
8A					+				
8B		+	+		+	+			APEC 3
9A									
10A		+	+	+	+				APEC 4
11A									
12A	+		+		+				
13A	+				+				
13B		+	+		+	+			APEC 5

14A									
15A		+	+		+		+	+	APEC 6
16A					+				
17A									
17B		+	+		+	+			APEC 7
18A	+								
19A		+	+		+				
20A									
20B		+	+		+	+			APEC 8
21A			+						
21B		+	+		+	+			APEC 9
22A		+	+		+				
22B		+	+	+	+	+	+		APEC 10
23A		+				+			
23B		+	+	+	+	+	+		APEC 11
24A		+			+				
24B									
25A	+		+		+				
26A		+							
27A					+				
27B		+	+		+	+			APEC 12
28A	+				+				
29A		+	+		+				
29B		+	+		+	+			APEC 13
30A	+								

A total of 17% of the isolates do not possess any virulence gene, 26% with one virulence genes, 12% with two virulence genes, 14% with three virulence genes, 24% with 4 virulence genes, 2% with 5 virulence genes and 5% with six virulence genes. According to Thomrongsuwannakij et. al. (2020), *E. coli* can be classified as APEC if it harbour at least four out of eight virulence genes. Hence in total, only 13 (31%) out of 42 isolates were classified as APEC. These APEC isolates came from 13 different chickens.

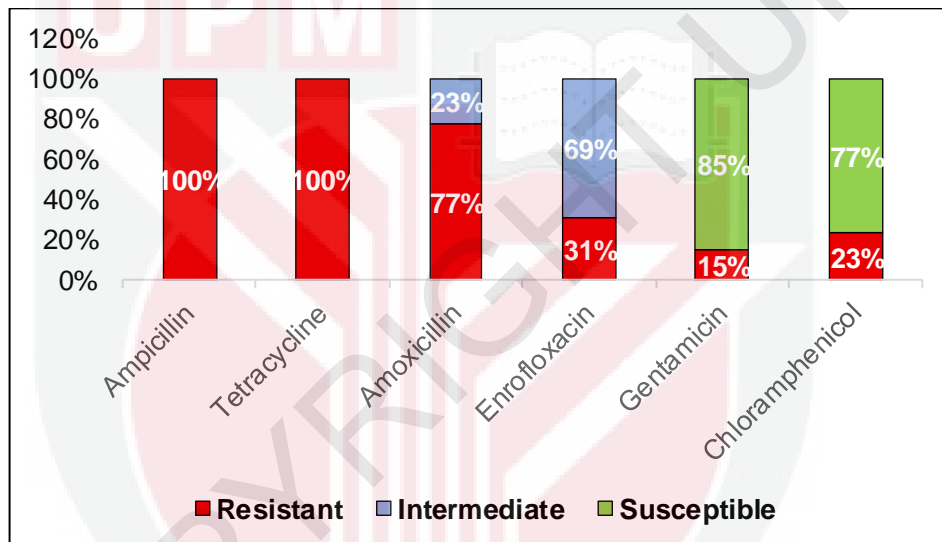
Figure 13: Virulence Associated Genes of *E. coli* isolates

From the 13 APEC isolates identified, *iucD* was the most prevalent gene present as it was detected in all 13 isolates. Second most prevalent gene were *irp2* and *iss* as both genes were found in 12 out of 13 APEC isolates. Besides that, *tsh* was identified in 10 out of 13 APEC isolates, four for *papC*, three for *vat*, two for *cva/cvi* and only one was found for *astA* as shown in Figure 14.

Figure 14: Virulence Associated Genes in 13 APEC isolates

The AST results that was conducted for the APEC isolates showed 100% resistance against ampicillin and tetracycline while 77% of the isolates were resistant to amoxicillin. A high intermediate of 69% was observed for enrofloxacin while 85% and 77% of susceptibility were recorded for gentamicin and chloramphenicol respectively (Figure 15).

Figure 15: Antibiotic Sensitivity Test of APEC isolates



5.0 DISCUSSION

Escherichia coli was obtained from all 30 chickens (100%) that were sampled. This result is as *E. coli* are normal microflora that is present in the chicken's intestines (Nolan, 2023). Cloacal swabbing sampling method used in this study increases the chances of obtaining *E. coli* from the chickens. This was reported in few studies that had successfully isolated 88.3% up to 100% of *E. coli* from cloacal swabbing collection method (Ngai et al., 2021; Lawwyne et al., 2019). In contrast, a study by Ferdous et al. (2023), showed that there were only 43% and 51% occurrence of *E. coli* isolated from tracheal and oropharyngeal swabbing of clinically sick chickens which were much lower in number compared to the results using a cloacal swab sampling method.

Out of all 42 *E. coli* isolates obtained, 13 isolates were identified as APEC by virulence associated genes detection using PCR. In comparison to previous studies conducted in Malaysia, results regarding occurrences of APEC were varied. A study conducted in Veterinary Research Institute (VRI) had reported prevalence of 178 isolates identified as APEC out of 471 colibacillosis cases that was sent to the institute (Khoo et al., 2010). Besides that, another study had only reported five isolates out of 25 *E. coli* isolates that was able to classify as APEC (Faiz, 2012). Another study done by Syuhada et al. (2014) had observed an occurrence of 24% of APEC from the *E. coli* isolates obtained. Difference of results among studies can be attributed due to different sampling methods as some studies only used organs such as liver, heart and peritoneum while others have mixtures of pooled organs and cloacal swabs as sampling method. Aside from that, the location of sampling can also lead to difference of results gained. Prevalence study conducted by VRI had reportedly obtained its samples predominantly from Perak poultry farms while Faiz (2012) had reported to

obtain his samples mainly from poultry farms in Selangor. Samples for a study conducted by Syuhada et al. (2014) had been collected from broiler farms of three different states which were Johor, Melaka and Perak.

From 13 APEC isolates that had been identified, the most prevalent virulent gene detected was *iucD* as it was found in all 13 isolates. Aside from that *irp2* was the second most prevalent gene where it was only absent in 1 out of 13 isolates obtained. Both virulence genes are involved in the iron acquisition system of *E. coli* (Kathayat et al., 2021). Iron serves as a vital nutrient crucial for *E. coli* development, influencing various cellular processes such as oxygen transport, electron transfer and enzymatic functions (Gao et al., 2021). Hence, a successful strategy for iron uptake is crucial for the proliferation and infection of APEC in a chicken. In environments where iron is scarce, *E. coli* has adapted by possessing multiple virulent genes that participate in iron uptake, transport and utilization systems seen in this study where there were two virulent genes for this process.

Besides that, identification of *iss* virulent gene was as the same amount as *irp2* which was 12 out of 13 APEC isolates. According to Kathayat et al. (2021), *iss* virulent gene is involved in protectins production by APEC. This article further explains protectins, comprising bacterial capsules, outer membrane proteins, and lipopolysaccharide (LPS) components, play a crucial role in safeguarding APEC from the host immune system. These elements collectively contribute to shielding against phagocytic engulfment by macrophages and protect against bactericidal effects of the host serum mediated by the complement system. There are several genes that encode protectins, *iss* gene helps to increase the serum survival for APEC. In addition, protectins also involve in APEC adhesion, invasion and colonization of the targeted chickens.

Moving on to the virulent genes that are apart of adhesins production are *tsh* which are detected in 10 out of 13 APEC isolates and *papC* gene that could be observed in only 4 out of 13 APEC isolates. The process of adhesion or attachment to host cells serves a dual purpose for pathogens (Aleksandrowicz et al., 2021). Firstly, presence of adhesins virulent gene facilitates the utilization of appendages and providing motility for APEC. In addition, it acts as a defense mechanism, shielding them against potential host defenses and preventing their removal from the host surface. All these attributes enable the introduction of the APEC effector proteins into the healthy chicken cells.

Lastly, only few APEC consists of *vat* and *astA* which is known to be under toxins genes (Kathayat et al., 2021). Toxins genes helps the APEC to invade and causes tissue damage of the host. According to Paixao et al. (2016), APEC secrete vacuolating autotransporter toxin (*vat*) much more often than arginine succinyltransferase (*astA*) which is a heat-stable enterotoxin. As a result, the prevalence of *vat* in the APEC isolates obtained were more than *astA*.

Antibiotic sensitivity test was done using six commonly used antibiotics registered for broiler chicken farms use in Malaysia. The results obtained in this study were mainly compared to the recent report from the Department of Veterinary Services (DVS), Malaysia on the National Antimicrobial Resistance (AMR) surveillance (2021) and few other studies. Firstly, this study had obtained 100% resistance against ampicillin and tetracycline. In comparison, DVS had similarly reported 100% resistance and there was another study had obtained slight decrease of 87% resistance (Ibrahim et al., 2021) against ampicillin. For tetracycline, DVS data was lower (82%) than this study obtained while Ibrahim et al. (2021) obtained only few differences of 94.6% resistance. There was also a high resistance of 77% for

amoxicillin which were from the same group of drugs with ampicillin. A study conducted in Thailand had obtain slight similar results of 70.2% resistant of APEC isolates they obtained towards amoxicillin (Thomrongsuwannakij et al., 2020) while other studies previously mentioned had not included amoxicillin in their reports. Results for enrofloxacin were different as this study results shown to be high intermediate of 69% while data report by DVS was resistance of 86%. DVS reported to had 56% resistance towards gentamicin while in contrast this study had obtained only 15% resistance which is quite similar to Ibrahim et al. (2021) data that obtained 20.2% resistance for same antimicrobial drug. However, the greatest difference observed was the results for chloramphenicol where both DVS and Ibrahim et al. (2021) reports showed high resistance of 91% and 84.5% respectively while this study obtained only 23% resistance and the remaining 77% was susceptibility. This difference may be due to the samples included in the study; samples from this study uses samples from one single farm while the DVS report and other studies is based on a proper surveillance sampling plan and carried out in multiple farms. It is clear that the AST results in this study does not represent the whole country, however it adds in the data of the susceptibility of the *E. coli* in the country.

The development of high antibiotic resistance in the treatment of avian colibacillosis poses a significant threat to the poultry industry. This scenario renders the use of antibiotics ineffective and redundant. Therefore, adopting a prudent approach in the use of antimicrobial agents is crucial for the effective treatment of animal diseases, aiming to mitigate the escalation of bacterial resistance in poultry industry. In addition to antimicrobials, alternative strategies, such as the creation of vaccines against APEC should be explored and developed to provide viable alternatives for disease control in poultry.

6.0 CONCLUSION

In conclusion, the study revealed a 100% occurrence of *E. coli* in the 30 sampled chickens with the detection of 31% of APEC found from all 42 *E. coli* isolates obtained. In addition, identification of 13 APEC isolates highlighted the prevalence of the *iucD* virulent gene which is crucial for the iron acquisition system known to be vital for APEC development. In contrast, toxin genes *vat* and *astA* were less prevalent but contributed to APEC's ability to invade and cause tissue damage in the host. All the obtained APEC isolates were multiple drug resistant bacteria with resistance against penicillin, tetracycline and fluoroquinolone.

7.0 LIMITATIONS

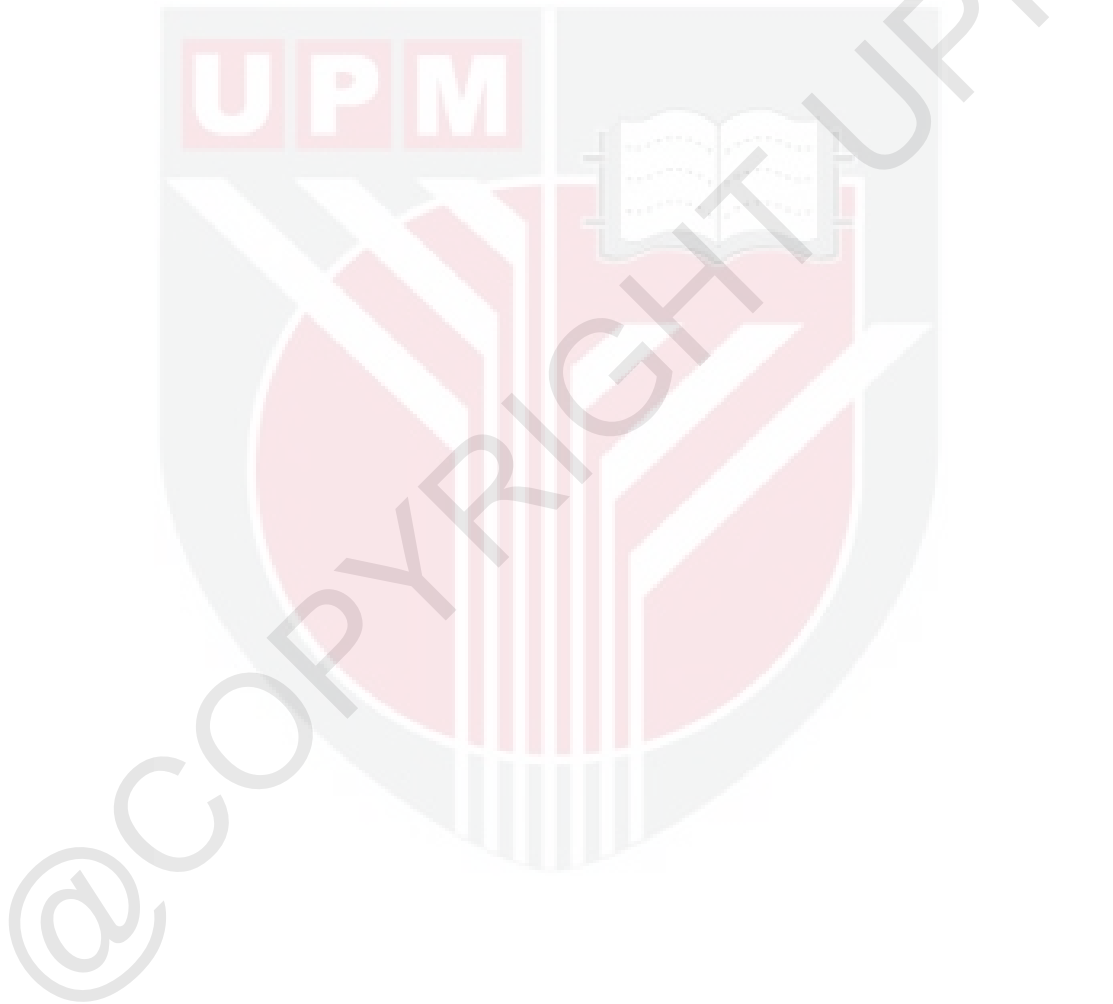
To enhance the robustness of future studies, several key considerations should be considered. Firstly, an expansion of the sample size is imperative to ensure a more comprehensive representation of the entire farm population. Including samples from multiple farms will help to capture the diversity of microbial populations in different environments. In addition, it is essential to gather detailed information about the farm's history and management practices. Understanding the types of antibiotics previously used from the farm and the overall disease management strategies used can provide valuable insights into the potential factors influencing bacterial resistance patterns. Furthermore, diversifying the types of samples collected beyond cloacal swabs for example collecting pooled organs can yield a broader spectrum of *E. coli* isolates. Thus, it can help to get a more comprehensive examination of APEC occurrence and allows for a deeper exploration of its virulent genes distribution.

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9.0 APPENDIX

Antibiotic Sensitivity Test Results

Zone of inhibition

ANTIMICROBIAL AGENT / I.D.	AMP 10	AML 10	CN 10	ENR 5	TE 30	C 30
APEC 1	0	16.0 mm	20.0 mm	6.6 mm	6.4 mm	19.7 mm
APEC 2	0	0	21.8 mm	7.0 mm	0	0
APEC 3	0	16.6 mm	19.3 mm	21.9 mm	0	21.7 mm
APEC 4	0	0	9.5 mm	0	0	0
APEC 5	0	13.3 mm	19.8 mm	21.5 mm	0	18.8 mm
APEC 6	0	0	7.7 mm	22.0 mm	5.7 mm	25.7 mm
APEC 7	0	0	22.5 mm	17.5 mm	6.9 mm	22.0 mm
APEC 8	0	12.8 mm	20.2 mm	22.0 mm	0	22.9 mm
APEC 9	0	16.6 mm	17.8 mm	20.0 mm	0	18.3 mm
APEC 10	0	0	19.4 mm	6.6 mm	0	19.1 mm
APEC 11	0	0	19.1 mm	20.0 mm	0	0
APEC 12	0	12.0 mm	19.2 mm	22.0 mm	0	18.0 mm
APEC 13	0	11.0 mm	16.7 mm	20.2 mm	0	18.6 mm