ISSN: 2395-6887

Contents lists available at www.iosi.in

ISOI Journal Of Microbiology, Biotechnology And Food Science

Volume 3 Issue 1; January-February-2017; Page No. 01-08

RESEARCH

Phenotypic and Molecular Characterization of *Escherichia coli* Isolates from Japanese quails (*Coturnixcoturnix japonica*) in Kelantan, Malaysia

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ARTICLE INFO

Received 10 Feb. 2017

Accepted 11 Mar. 2017

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Key words: Antibiotics, Coturnixcoturnix japonica, *E. coli*, Isolation, quails

ABSTRACT

This study was conducted to characterize *E. coli* isolates in *Coturnixcoturnix japonica* (Japanese quails). A total sample of 80 cloacal swabs was collected (40 from 2 months old quails and 40 from 3 weeks old quails). The isolates were confirmed using culture, microscopy and biochemical identification. Molecular typing using Polymerase chain Reaction (PCR) assay confirmed that 60 (75%) of the isolates to be *E. coli* and none of the isolates confirmed belonged *E. coli* 0157 serotype. Statistical analysis using Chi square showed no significant difference in prevalence of *E.coli* between the 2 months old and the 3 weeks old quails (P>0.05). Antibiotic susceptibility testing showed that there was high resistance of 75% of the *E. coli* isolates towards multiple antibiotics such as tetracycline and ampicillin while sensitivity of 75% was found for ciprofloxacin and gentamycin. Our findings suggests that *E. coli* infection may be endemic in the study area but serotype *E. coli 0157* may be absent. The multiple drug resistances dissipated in our study calls for concern in the face of increasing limited antibiotic options and more searh for genuine antibiotics in managing colibacillosis on Malaysian quails. Proper hygiene regime and biosecurity measures are necessary to minimize the risk of spread of infection to the human population.

INTRODUCTION

Quails are susceptible to some viral, bacterial and other infectious diseases caused by different pathogens (Odugbo et al., 2006;). *Escherichia coli* is a coliform bacterium belonging to the family *Enterobacteriaceae* and is well known to be widely distributed in the intestinal tract of animals, birds and humans (Frederick, 2011, Quinn et al., 2004). Avian colibacillosis caused by *E.coli* is considered to be a major disease in the poultry industry worldwide, which affects all ages and breeds of poultry (Salehi and Ghanbarpour, 2010).This disease causes economic losses resulting high morbidity, mortality and decreased in productivity of the affected birds (Lutful, 2010) It also causes yolk sac infection, omphalitis, respiratory infection, swollen head syndrome, septicaemia, polyserositis, enteritis, cellulitis and salpingitis (Lutful, 2010). Colibacillosis of poultry is characterized in its acute form by septicaemia resulting in death and in its sub acute form by pericarditis, air sacculitis and peri hepatitis (Lutful, 2010).

The poultry sector is the largest of all the livestock industries in Malaysia and is practiced at levels ranging from subsistence to large scale commercial operation (Othman et al., 2014). Poultry meat and eggs consumption is widespread and considered inexpensive, and they also fulfill the preference of all ethnic groups without religious restriction. Quail meat in some countries is considered as beneficial food for all ages due to its high meat yield, little shrinkage during cooking, fast cooking and also low level of cholesterol. Quail meat is also a perfect source of vitamin B6, niacin, thiamine, pantothenic acid and riboflavin, so it is considered a favorite among other species of poultry meat (Hamad et al., 2012). The use of contaminated poultry meat and poultry products poses a health risk of contracting foodborne diseases, which has been given little attention in terms of epidemiological

studies. Food borne diseases are an important public health problem, producing a substantive impact on the economy and trade in Malaysia and other developing countries. Epidemiological reports suggest that poultry meat is still the primary cause of human food poisoning (Mulder. et al., 1999). Poultry meat is more popular in the consumer market because of advantages such as easy digestibility and acceptance by the majority of people (Yashoda et al., 2001). Nevertheless, the presence of pathogenic and spoilage microorganisms in poultry meat and its byproducts remain a substantial concern for suppliers, consumers and public health officials worldwide. In Malaysia, data on prevalence of certain food borne pathogen such as *Salmonella*, *Listeria*, *Staphylococcus*, *Campylobacter* and *E.coli* are available for most animals except quails (Adzitey et al., 2010). It showed that the prevalence of *Escherichia coli* reported in Malaysia ranged from 22.6 to 88.0% (88% in ducks with pathogenic strain *O157* at 2% and beef, 36%).

Malaysian consumers, as found in most developed nations are also becoming increasingly aware and concern about food safety issues. *Escherichia coli* have been consistently linked with food borne illnesses in many nations of the world. Pathogenic strains of *E.coli* when consumed could cause gastrointestinal illness in healthy humans and animals (Awadallah et al., 2013). Since the quail industry is a growing industry in Malaysia with a soaring demand for quails at 20-25% since 1995, not many studies have been conducted on *E. coli* in quails. To the best of our knowledge, there is no published report on the isolation of *E.coli* in quails in Kelantan, Malaysia. Our study is therefore the first to determine phenotypic and molecular characterization of *E. coli* in quails in selected farms in Kelantan Malaysia with the view of determining the antibiotic resistance patterns of the isolates to commonly used antimicrobial agents.

Materials and methods

Study area

Kota Bharu, Kelantan state is located in the East coast of Peninsular Malaysia (06°10'N and102°20'E). Out of three available quail farms in the state, one was identified as a commercial farm that serves the community located atKota Bharu, the state capital that has a human population of 468, 438 (Khan et al., 2014). The population of quails in this farm was 6000 birds.

Collection of Sample and Sampling

The sample size was calculated based on the reported prevalence of 5.7% from a study in Italy (Dipineto et al., 2014), due to the fact that no prior studies of this nature have been carried out in Malaysia, using Epi Info version 7 (Dean et al., 1990). Purposive sampling was carried out and 80 cloacae swabs were taken aseptically from 3 weeks old quail and 2 months quails. The number of cloacal swabs was recorded as sample of 3 weeks old quails (n=40) and 2 months old quails (n=40). Each swab was placed in a bottle containing Buffered Peptone Water (BPW) as pre-enrichment medium and placed straightaway into an ice box with a temperature of 4^{o}C after collection. The sample was brought to the Bacteriology laboratory at Faculty of Veterinary Medicine, Universiti Kelantan, Malaysia . The samples were placed in BPW and incubated at37^{o}C for 24 hours.

Isolation of bacteria in pure culture

A loop full of rinse incubated cloacal swab sample that was placed in the Buffer Peptone Water (BPW) was inoculated onto MacConkey agar and incubated overnight at 37^{o}C.The overnight bacterial colonies that were lactose positive on MacConkey agar were then streaked onto Eosin Methylene Blue (EMB) agar and incubated at 37^{o}C for 24 hours. Single colonies were further sub-cultured to nutrient agar until pure cultures were obtained (Cheesbrough, 1984).

Identification of bacteria

Identification of bacteria was performed on the basis of colony morphology, Gram's staining and biochemical tests (Cheesbrough, 1984). Colony characteristics such as size, shape, surface texture, edge, elevation and colour development after 24 hours incubation at 37^{o}C was recorded. A single colony of bacteria was picked up with inoculation loop and it was smeared on a sterile glass slide and gram stained and examine under light microscope to observe the morphology of bacteria (Quinn et al., 2004).

Biochemical tests

The biochemical tests conducted included: TSI Agar slant test, catalase test, Methyl red test, Voges-Proskauer test and Indole test. The isolates were then stored in nutrient agar slants for further studies.

Polymerase Chain Reaction (PCR)

This test is more efficient, rapid, sensitive and reliable for detecting and genotyping *E.coli* species (Gomes et al., 2005). A pure bacterial colony was mixed with 200 µl of PBS which was boiled for 10 minutes and then immediately kept on ice for 10 minutes. Finally centrifugation was done at 10000 rpm for 10 minutes. The supernatant was collected and used as DNA template for PCR. Oligonucleotide sequences of primer used in the detection of housekeeping gene (*pho*A) *E.coli* and Enterohemorrhagic *E.coli* primer were used to detect *rfb* O157 gene (Table 1). PCR product was separated by electrophoresis on 1.5 % (w/v) agarose gels in Tris-borate-EDTA buffer along with a 100bp DNA ladder. Fragments were visualized by UV illumination and photographed after ethidium bromide staining.

Table 1: Primer used in the study

Primer	Target gene	Primer sequences (5' → 3')	Amplification products (bp)	References
Pho-F	Alkaline phosphate (*pho*A)	AAGCCCGGACACCATAAATGCCTG TTCTGG	903	(Kong et al., 1999)
Pho-R	Alkaline phosphate (*pho*A)	GGTTGGTACACTGTCATTACGTTGC GGATT	903	(Kong et al., 1999)
O157 F	nt* 393-651 of *rfb*$E_{O157:H7}$	CGGACATCCATGTGATATGG	259	(Paton and Paton, 1998)
O157 R	nt* 393-651 of *rfb*$E_{O157:H7}$	TTGCCTATGTACAGCTAATCC	259	(Paton and Paton, 1998)

*nt- nucleotide.

Antibiotic Sensitivity tests

Antibiotic sensitivity test was performed on *E. coli* isolates using the disk diffusion technique which was carried out using Mueller Hinton agar. Interpretation of zones of inhibition diameter was carried out according to standard procedures of the Clinical and Laboratory Standards Institute, 2007 (CLSI, 2007).

Data analysis

The data collected in this research was analysed using descriptive statistics and Chi Square in SPSS Windows, Version 22.0 (SPSS, 2013).

RESULTS

The overnight bacterial colonies that were lactose fermenters, pink colonies with a surrounding darker pink area of precipitate bile salt on MacConkey agar were identified. Sixty samples were lactose fermenters. The growth of *E.coli* on EMB agar was indicated by smooth, circular, black or green colour colonies with metallic sheen. 60 out of 80 samples show metallic green sheen. A thin smear prepared from the colony on EMB agar stained by Gram's staining revealed Gram negative, short rod shaped bacteria, arranged in single paired or in short chain under microscope. The biochemical reactions dissipated by the *E.coli* isolates from quails in Malaysia were shown (Table 2 and 3).

Table 2: Biochemical tests of *E.coli* isolated from 3 week old Quails

Age group of Quails	Name of biochemical test	Results	Interpretation	Sample positive	Sample negative
3 weeks old n = (40 cloacal swabs)	Oxidase	Negative	*E.coli*	40	0
	Catalase	Positive	*E.coli*	40	0
	TSI (Triple Sugar Iron) test	A/A, break in medium and formation of bubble	*E.coli*	40	0
	Citrate	Negative	*E.coli*	30	10
	Urase	Negative	*E.coli*	30	10
	SIM (Sulfur Indole Motility)	Positive S - H2S formation I – red ring (+) M – positive	*E.coli*	40	0
	MR	Positive (Change to pink colour)	*E.coli*	40	0
	VP	Negative (pink round ring)	*E.coli*	40	0

Table 3: Biochemical test of *E.coli* isolated from 2 months old Quails

Age group of Quails	Name of biochemical test	Results	Interpretation	Sample positive	Sample negative
2 months old n=(40cloacal swabs)	Oxidase	Negative	*E.coli*	40	0
	Catalase	Positive	*E.coli*	40	0
	TSI (Triple Sugar Iron) test	A/A, break in medium and formation of bubble	*E.coli*	40	0
	Citrate	Negative	*E.coli*	30	10
	Urase	Negative	*E.coli*	30	10
	SIM (Sulfur Indole Motility)	Positive S - H2S formation I – red ring (+) M - positive	*E.coli*	40	0
	MR	Positive (Change to pink colour)	*E.coli*	40	0
	VP	Negative (pink round ring)	*E.coli*	40	0

Molecular detection of *E.coli*

DNA extracted from 60 *E.coli* isolates were used in the polymerase chain reaction (PCR) assay. A monoplex PCR detecting alkaline phosphate gene (*phoA* gene) was performed on all *E.coli* strains. All isolates showed positive results for the presence of *phoA* gene (903 bp), thus confirming their identity as *E.coli* (Figure 1 and 2).

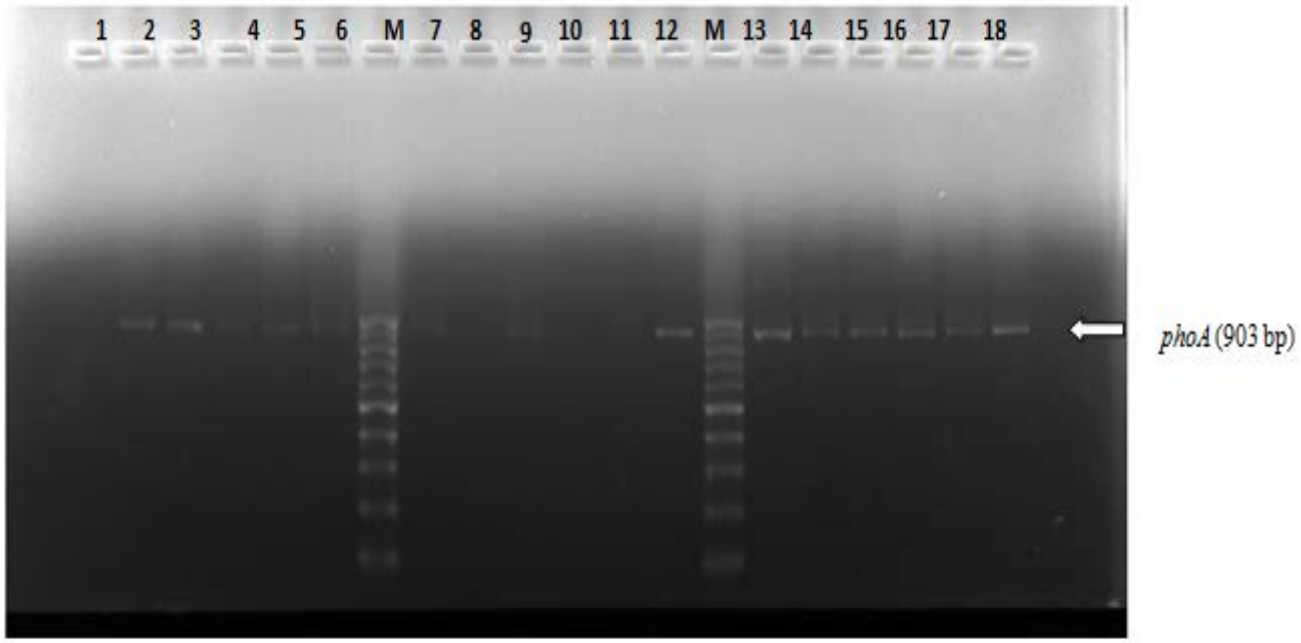

Fig 1: Sample from 3 weeks old Quails a representative gel of monoplex PCR for *phoA* gene (Housekeeping gene of *E.coli*) from the *E.coli* isolates. Lane M: 100 bpDNA. Ladder 1 to 18 sample showing positive results (*E.coli* spp).

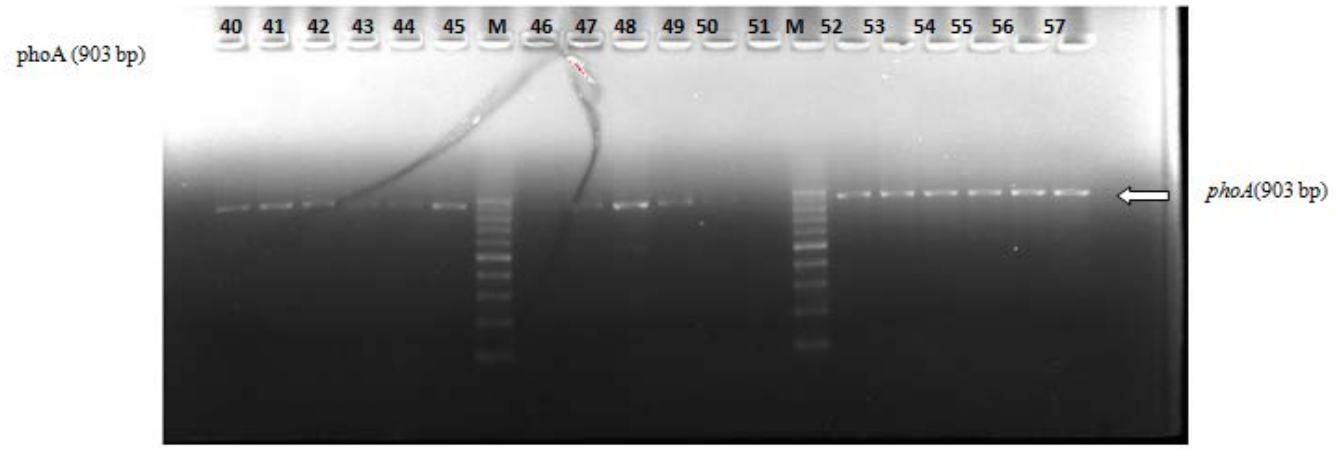

Fig 2: Sample from 2 months old Quails. A representative gel of monoplex PCR for *phoA* gene (Housekeeping gene of *E.coli*) from the *E.coli* isolates. Lane M: 100 bp DNA. Ladder 1 to 18 sample showing positive results (*E.coli* spp).

Molecular detection of Pathogenic *E.coli O157*

DNA extracted from 60 *E.coli* isolates were used in the polymerase chain reaction (PCR) assay. PCR detecting Enterohemorrhagic *E.coli* gene (rfb_{O157}) was performed on all *E.coli* strains. All isolates showed negative results for the presence of rfb_{O157} gene (259 bp), thus confirming there is no *E.coli* 0157 serotype (Figures 3 and 4).

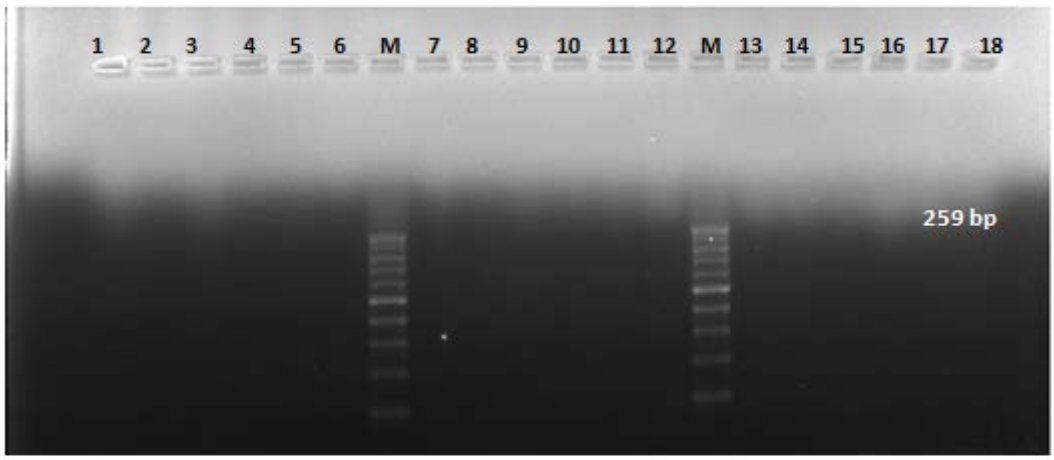

Fig 3: Sample from 3 weeks old Quails. A representative gel of PCR for rfb_{O157}gene (Enterohemorrhagic *E. coli*) from the *E.coli* isolates. Lane M: 100bp DNA. Ladder 1 to 18 sample showing negative results for *E.coli* O157.

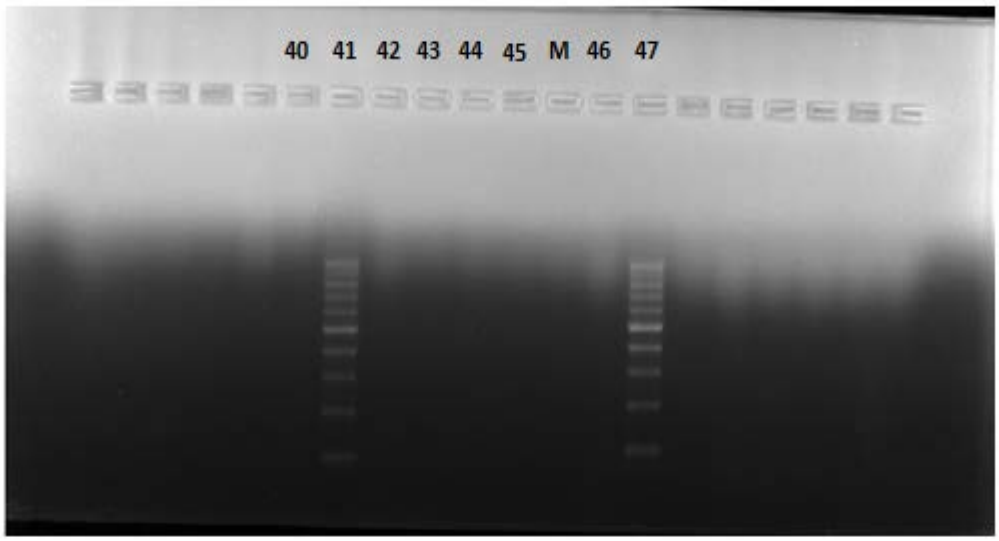

Fig 4: Sample from 2 Months old Quails. A representative gel of PCR for rfb_{O157}gene (Enterohemorrhagic *E.coli*) from the *E. coli* isolates. Lane M: 100bp DNA. Ladder 40 to 57 sample showing negative results for *E.coli* O157.

Prevalence of *Escherichia coli* species by farm district

There was no significant difference (P>0.05) using Chi-sqaure analysis between the 3weeks old quails and 2 months old quails for *E.coli* positivity. The overall prevalence of *E.coli* was 75 %.Ciprofloxacin and Gentamycin showed high sensitivity towards *E.coli* at 75% and the results are similar in 3 weeks old quails and 2 months quails in the same farm. Streptomycin showed intermediate resistance towards *E.coli* of about 50% in 3 weeks old quails and 25% in 2 months old quails(Table 4 and 5)

Table4: Results of antibiotic susceptibility test for 3 weeks old Quails.

Age of Quails Results (3 weeks old) n=40 sample	**Zone of inhibition (mm) for AMP 10 µg**	**CIP 5 µg**	**CN 10 µg**	**S 10 µg**	**TE 30 µg**
Sensitive (n)	0	30	30	0	0
Percentage sensitive (%)	0%	75%	75%	0%	0%
Intermediate (n)	0	0	0	20	0
Percentage intermediate (%)	0%	0%	0%	50%	0%
Resistance (n)	30	0	0	10	30
Percentage resistance (%)	75%	0%	0%	25%	75%

AMP=Ampcillin; CIP= Ciproflaxacin; CN=Gentamicin; S=Streptomycin; TE= Tetracycline

Table 5: Results of antibiotic susceptibility test for 2 months old Quails.

Age of Quails Results (2 months old) n=40 sample	**Zone of inhibition (mm) for AMP 10 µg**	**CIP 5 µg**	**CN 10 µg**	**S 10 µg**	**TE 30 µg**
Sensitive (n)	0	30	30	0	0
Percentage of sensitive (%)	0%	75%	75%	0%	0%
Intermediate (n)	0	0	0	10	0
Percentage of intermediate (%)	0%	0%	0%	25%	0%
Resistance (n)	30	0	0	10	30
Percentage of resistance (%)	75%	0%	0%	25%	75%

AMP=Ampcillin; CIP= Ciproflaxacin; CN=Gentamicin; S=Streptomycin; TE= Tetracycline

Discussion

Avian colibacillosis caused by *E.coli* is a major concern and major health problem in the poultry industry in Malaysia due to the likely threat of the bacterium causing food borne illness. In this study, an attempt was made to determine the prevalence of *E.coli* species and pathogenic *E.coli* in Quails in a commercial quail farm. The detection of *E.coli* was conducted by using both phenotypic and molecular techniques.

The total prevalence of Colibacillosis in the selected Kelantan Quails farm was 75 % of the total sample collected (80 samples). According to age of quails, 3 weeks old and 2 months old have same percentage of *E.coli* as indicated by our findings. Japanese quail were reported to be resistant to many diseases (Roy et al., 2006). However, the high prevalence of *E. coli* should

be expected as it is an innocuous resident of the gastrointestinal tract that is ubiquitous but yet capable of causing many infections around the world in both humans and animals (Croxen et al., 2013).

The non-isolation of *E. coli* 0157 agrees with a study in poultry over a one year period in which no *E. coli* 0157 was found in 1000 chickens indicating that this strain may be rare in poultry and where found prevalence is usually as low as 1.5% (Doyle and Schoeni, 1987). Previous studies have shown that quails have been infected with *E.coli* O9 serotypes in India. Other strains of *E.coli* such as O128 and O26 serotypes in Italy (Dipineto et al., 2014) which were not specifically tested for in this study. There are no statistical significant differences with reference to age of birds and the isolation rate of *E.coli* perhaps because *E.coli* is a commensal bacteria that is normally present in intestines of birds of any age.

Molecular identification of *E.coli* revealed that the isolated organisms are definitely *E.coli* bacteria using the *phoA* gene (903bp) which is common gene sequence for housekeeping *E.coli* to all the samples in 3 weeks old quails and 2 months old quails. But the PCR revealed that the isolated *E.coli* was negative against the rfb_{O157} gene (259bp) pathogenicenterohemorrhagic *E.coli.* It may be concluded that the pathogenicity of the isolated bacteria is inconclusive or it may use other types of toxin for pathogenesis.

Antibiotic susceptibility profile of *E.coli* showed high resistance to multiple drugs such as Tetracycline and Ampicillin but sensitivity towards Ciprofloxacin and Gentamicin. Colibacillosis is endemic in the study area but pathogenic colibacillosis caused by *E.coli* 0157 is not endemic in the area. Antibiotics (Ciprofloxacin and Gentamicin) could be used for the treatment of clinical case of colibacillosis in quails. Strict biosecurity measures should be implemented to prevent this disease in quails in future.

The various *E.coli* isolates showed high resistance to multiple drugs such as Ampicilli (AMP) and Tetracycline (TE). The results also show that *E.coli* is sensitive to Ciproflaxacin (CIP) and Gentamicin (CN) and shows intermediate sensitivity to Streptomycin (S). There have been several studies in which indicated many *E.coli* isolates in chickens were found resistant to Tetracycline and Ampicillin but fewer studies in Quails (Apata, 2009). The five commercial antibiotics chosen for this study shown in are widely used in Malaysian farms as the first line of treatment and certain antibiotics are also used as growth promoters in poultry sectors. Out of 5 antibiotics tested, high resistance level were detected for Ampicillin (75%) and Tetracycline (75%) which may be due to widespread misuse of antibiotics in livestock and use of sub-therapeutic dosages (Van den Bogaard et al., 2001). These results are similar in both 3 weeks old quails and 2 months quails indicating that age may not necessarily play a significant role in development of antibiotic resistance in quails.In Malaysia, antibiotics have been used as feed addictives to improve feed efficiency and weight gain in poultry sector. Many antibiotics are also used in feed and water to control disease as prophylactics (Jahantigh et al., 2012) . Indiscriminate use of antibiotics has provided selective pressure for the emergence of multi drug resistance strains of bacteria associated with poultry products. In this study, the *E.coli* isolates showed high resistance (75%) to multiple drugs such as Tetracycline and Ampicillin but were sensitive towards Ciprofloxacin and Gentamicin. Comparing data to other poultry such as chickensthe antibiotic resistance appeared to show similar patterns to that found in quails in this study (Apata, 2009).

Conclusions

Based on this study, and from the findings of our present study, it may be concluded that colibacillosis is endemic in the study area but pathogenic colibacillosis caused by *E.coli* 0157 is not endemic in the area. Though the prevalence rate of other strains of *E. coli* was 75%. There is high potential risk of infection to susceptible individuals. Our findings also showed no statistical significant differences in age of birds and the isolation of *E.coli.* Molecular identification using the *phoA* gene (903bp) revealed common gene sequence for housekeeping *E.coli*. Antibiotic susceptibility profile of *E.coli* showed multiple resistances to drugs such as Tetracycline and Ampicillin but sensitivity towards Ciprofloxacin and Gentamicin. Strict biosecurity measures should be implemented to prevent this disease in quails. It is also necessary to investigate quials farms in Kelanthan for other microorganisms which may affect the economics of quail production in Malaysia.

Acknowledgment

The authors would like to thank the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Malaysia for funding this study as part of its research and teaching services for January to June 2015. The authors also wish to thank Assoc.Prof. Dr. Aung TunKhaing for his help in ensuring the success of the sampling process.

Conflict of interest

The authors declare and attest that they have no conflict of interest.

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