PHYLOGENETIC GROUPING AND VIRULENCE GENE PROFILES OF *Escherichia coli* ISOLATED FROM CHICKEN

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ABSTRACT. Colibacillosis is a disease caused by avian pathogenic *E. coli* (APEC) and is one of the principle cause of morbidity and mortality in poultry worldwide which is represented by a complex syndrome characterized by multiple organ lesions. This study was carried out to determine phylogenetic grouping and virulence associated genes contained by *E. coli* isolates which is related in causing disease in chicken. *E. coli* isolates obtained from clinical cases of Veterinary Research Institute were re-identified by conventional methods. Phylogenetic grouping of the isolates was determined by triplex polymerase chain reaction (PCR), and the presence of eight virulence genes were identified by multiplex PCR. A total of 125 *E. coli* isolates were subjected to analysis of phylogenetic background and virulence associated genes profiling. Phylogenetic analysis demonstrated that most of the *E. coli* isolated from chicken in this study belonged to group B1 (36.0%), group D (28.0%), group A (27.2%) and group B2 (8.8%). Multiplex PCR analysis demonstrated that 96 (78.6%) of the *E. coli* isolates harbored at least one virulence gene, while 29 (23.3%) did not contain any virulence genes tested. The most prevalent virulence genes identified were *iss* (51.2%), followed by *iucD* (36.0%), *tsh* (32.8%), *vat* (16.0%), *astA* (13.6%), *irp2* (11.2%), *papC* (9.6%) and the least is *cva/cvi* gene (0%). None of the isolates harbored more than four virulence genes. Each of phylogenetic groups presented with different combinations of virulence genes, with no specific combinations of virulence genes found to correlate with *E. coli* phylogroups. None of the *E. coli* isolates harbored more than four virulence genes, suggesting that *E. coli* isolates from chicken in this study appear to be derived from commensal strains and may relate to environmental predispose factors especially stress factors in the host to establish infection.

Keywords: E. coli, phylogenetic group, virulence genes, PCR

INTRODUCTION

Avian pathogenic *E. coli* (APEC) is the aetiological agent for colibacillosis, a complex syndrome characterized by

multiple organ lesions including air saccultis, pericarditis, salphingitis, synovitis or yolk sac infection in poultry (Ewers *et al*., 2004; Johnson and Russo, 2002). Colibacillosis initiate through infection of respiratory tract frequently followed by septicemia, which is responsible for high economic losses (Ewers *et al.,* 2004).

The infection is usually initiated by predisposing agents such as biological factors including viral and mycoplasmal infection and environmental stresses (Barnes, 2003; Mbanga *et al*., 2015). Therefore, the disease is always considered as opportunistic infection, which causes secondary infection in poultry (Barnes, 2003; Hiki *et al*., 2014).

Previously, management approaches have been suggested to assist in disease control in farms, by protecting poultry against predisposing conditions, however this proved to be largely ineffective in controlling the disease (Mbanga *et al.,* 2015). Therefore, a treatment with antibiotics remains important to assist in reducing both incidence and mortality associated with the disease (Mbanga *et al*., 2015). Recently, there is increasing evidence that avian *E. coli* is becoming more resistant to antibiotics with high resistant rate towards certain antibiotic which is commonly used in therapeutic purposes in poultry farms (Wang *et al.,* 2013; Ahmed, 2013). Emergence of antibiotic resistance among avian *E. coli* strains allowed the bacteria to lose their potency as the first line of defense in treatment, thus making it more difficult to treat the infection. Introduction of vaccine approaches for control of disease outbreaks is desirable, however currently available vaccines are not totally effective (Schouler *et al*., 2012). Numerous studies have demonstrated that avian *E. coli* strains constitute a diverse characteristic, which prevents the identification of common properties that are useful for diagnostic methods and vaccination purposes. Conventional diagnostic methods such as classical serological method are also unable to identify virulence properties of *E. coli* strains, thus making it difficult to differentiate either pathogenic or non-pathogenic strains (Schouler *et al*., 2012).

Several studies demonstrated that certain virulence factors have been associated with virulence of *E. coli* in poultry, which is essential as potential for vaccine candidates. Virulence factors including those encoding for adhesion, toxins, iron acquisition systems, autotransporters, sugar metabolisms, serum resistance proteins, capsule and lipopolysaccharides complex have been identified as important factors correlated with virulence of avian *E. coli* strains (Ewers *et al*., 2005; Schouler *et al*., 2012; Mbanga *et al*., 2015). Distribution of those virulence factors among different countries are varied. Recently, no specific virulence associated genes contributing to pathogenecity of avian *E. coli* can be used as vaccine candidates in disease treatment (Van der Westhuizen and Bragg, 2012). Concrete information regarding

phylogroups and virulence genes profile of avian E.coli strains in the country is not available elsewhere. Therefore, the aim of this study is to determine the phylogenetic grouping and virulence associated gene profile of *E. coli* isolated from chicken in the country. This study may aid in characterization of avian *E. coli* strains which are important as baseline information for future vaccine or alternative treatment and development of diagnostic tools.

MATERIAL AND METHODS

Preparation of *E. coli* cultures

E. coli stock culture collection was obtained with approval from Veterinary Research Institute. All the isolates were from *E. coli* infection cases in broiler and village chicken with profuse growth on agar medium. The isolates have been identified and classified by conventional methods according to standard protocols. (Antigenic Formulae of Salmonella Serovars, WHO Collaborating for Reference and Research for Salmonella, 2007). The isolates were kept in maintenance medium until further tests.

Multiplex PCR for detection of virulence associated genes

Deoxyribonucleic acid (DNA) preparation

E. coli isolates were subcultured on 5% defibrinated blood agar and the plates were incubated at 37 °C for 18 to 24 hours. Few *E. coli* colonies were inoculated in Brain Heart Infusion (BHI) broth and incubated overnight at 37 °C. DNA was extracted by using a commercial kit (Promega ® SV Genomic Purification Kit) according to the manufacturer's protocols.

Multiplex PCR

E. coli isolates were examined for eight virulence genes including *astA, papC, irp2, vat, tsh, cva, iss* and *iucD* according to published primers (Ewers *et al.*, 2004). The primers used in this study were obtained from Integrated DNA Technologies, and are listed in Table 1.

Triplex PCR

The phylogenetic group (A, B1, B2 and D) of each isolate was determined by triplex PCR as described by Clermont *et al.* (2000), as listed in Table 2. Three pairs of primers for triplex PCR encoding of three markers including (i) *chuA* which amplify a gene required for heme transport; (ii) yjaA, a gene initially identified in the recent complete genome sequences of *E. coli* K-12 strain and (iii) TSPE4.C2 from

published subtractive library (Bonacorsi *et al*., 2000).

Ten microlitres of each DNA sample were added to the reaction mixture containing 0.1 µl of each primer pair (100 pmol, Integrated DNA Technologies), 10 µl 5× Bioline My Taq Reaction Buffer (15 mM magnesium chloride and 5 mM dNTPs) and 0.5 µl MY Taq DNA Polymerase (Bioline). The remaining portion was filled with sterile nuclease-free water up to 50 µl. Amplification was performed in 50 µl reaction using Eppendorf Mastercycler nexus Gradient GSX1 Thermal Cycler under the following conditions. For multiplex PCR for detection of virulence genes; initial denaturation step at 94 °C for 3 minutes, followed by 25 cycles of denaturation step at 94 °C for 30 second, annealing step at 58 °C for 30 second, extension step at 68 °C for 3 minutes, and final elongation step at 72 °C for 10 minutes. For triplex PCR for determination of phylogenetic group,

Table 1. Sequence of multiplex PCR primers and their product sizes

Name of gene	Primer	Primer sequence (5' – 3')	Size (bp)
enteroaggregative toxin	*astA*	TGCCATCAACACAGTATATCC TCAGGTCGCGAGTGACGGC	116
increased serum survival protein	*iss*	ATCACATAGGATTCTGCCG CAGCGGAGTATAGATGCCA	309
iron repressible protein	*irp2*	AAGGATTCGCTGTTACCGGAC AACTCCTGATACAGGTGGC	413
P fimbriae	*papC*	ATGATATCACGCAGTCAGTAGC CCGGCCATATTCACATAA	501
aerobactin	*iucD*	ACAAAAAGTTCTATCGCTTCC CCTGATCCAGATGATGCTC	714
Temperature sensitive haemagglutinin	*tsh*	ACTATTCTCTGCAGGAAGTC CTTCCGATGTTCTGAACGT	824
Vacuolating transporter toxin	*vat*	TCCTGGGACATAATGGTCAG GTGTCAGAACGGAATTGT	981
Colicin V plasmid operon	*Cva/cvi*	TGGTAGAATGTGCCAGAGCAAG GAGCTGTTTGTAGCGAAGCC	1181

Table 2. Sequence of triplex PCR primers and their product sizes

Gene		Primer sequence (5' – 3')		Size (bp)
ChuA.1	***ChuA.2***	GACGAACCAACGGTCAGGAT	TGCCGCCAGTACCAAAGACA	279
YjaA.1	***YjaA.2***	TGAAGTGTCAGGAGACGCTG	ATGGAGAATGCGTTCCTCAAC	211
TspE4C2.1	***TspE4C2.2***	GAGTAATGTCGGGGCATTCA	CGCGCCAACAAAGTATTACG	152

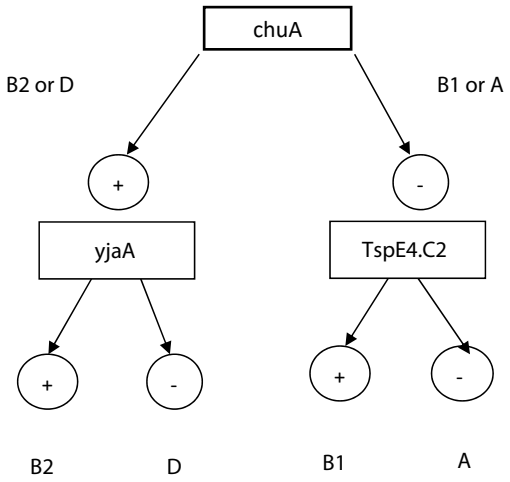

Figure 1. Dichotomous decision tree to determine the phylogenetic group of an *E.coli* strain by using the results of triplex PCR

the reaction was carried out under the following conditions: initial denaturation at 94.0 °C for 5 minutes, 30 cycles of (94.0 °C for 30 seconds, 55.0 °C for 30 seconds and 72.0 °C for 30 seconds) and final extension at 72.0 °C for 7 minutes.

The PCR products were visualized with 1.0% agarose gel (Hydragene ® Agarose powder) stained with 3 µl of Gel Green ® Nucleic Acid stain (Biotium). The PCR products were run along with 100 bp Hyperladder (Bioline) in TBE Buffer for 90 minutes at 60 volt before being visualised using Major Science gel documentation system. *E. coli* reference strains IMT 2740 were used as positive control for virulence genes study, and *E. coli* ATCC 8739 was for phylogroup analysis. Negative control

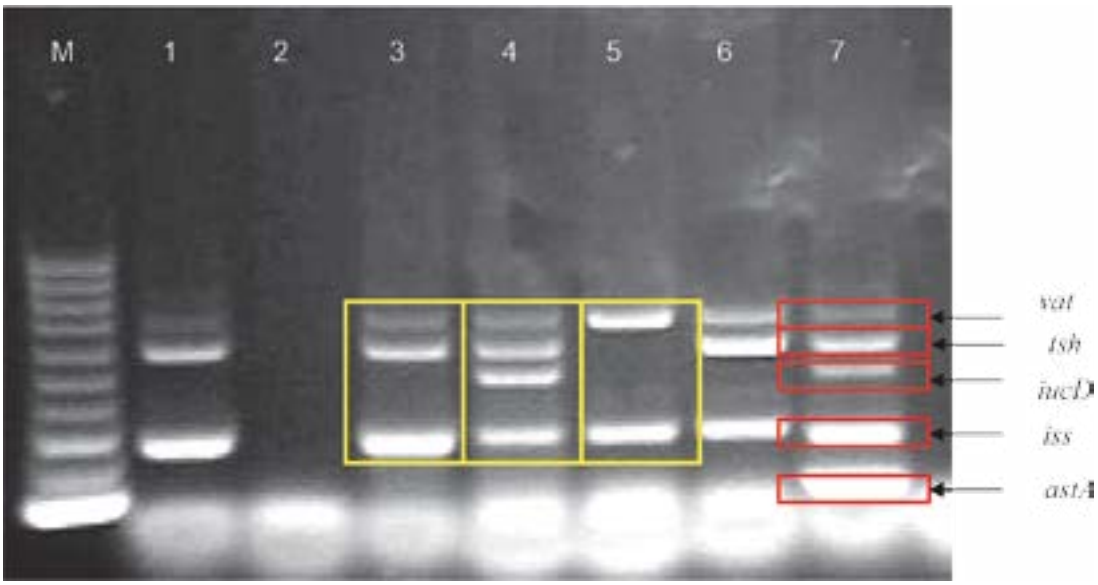

Figure 2. Agarose gel electrophoresis of the multiplex PCR products with representative *E.coli* isolates carrying different combinations of virulence associated genes. Yellow boxes show representative *E.coli* isolates with different number of virulence genes. Red boxes show expected bands of different virulence genes.

was sterile nuclease-free water. The PCR results were analyzed based on the established dichotomous decision tree as described by Clermont *et al*., 2000 (Figure 1) for phylogenetic grouping.

RESULTS

Results on distribution of virulence genes detected by multiplex PCR from *E. coli* isolates are shown in Table 3. Multiplex PCR analysis of the 125 *E.coli* isolates showed that at least 7 virulence genes were found among the examined isolates with different percentage (Figure 2). Among 8 virulence associated genes tested, the most prevalent is *iss* (51.2%), followed by *iucD* (36.0%) and *tsh* (32.8%). Other virulence genes detected include *vat* (16.0%), *astA* (13.6%), *irp2* (11.2%), *papC* (9.6%) while no *cva/cvi* gene (0%) was detected in all isolates.

Among the 125 *E.coli* isolates tested, 96 (76.8%) isolates harboured at least one virulence factor, while 29 (23.2%) isolates did not contain any virulence factor tested. Overall, 34 (27.2%) isolates contained three virulence associated genes, 27 *E. coli* isolates were found to harbor two virulence genes (21.6%) and only 8 (6.4%) contained four virulence associated genes. The results obtained showed that none of the *E. coli* isolates harbor more than four virulence associated genes.

Based on this finding, combination of virulence associated genes among the isolates varied based on the virulence genes they possessed. Among *E. coli* isolates which contain four virulence genes (VG), 2/8 (25%) harboured combination of *iucD-tsh-vat-iss* combination. Combination of *iucD-tsh-iss* (47.1%) and *iucD-iss* (25.9%) was the most frequently detected among those *E. coli* isolates containing at least three and two virulence genes tested respectively. *Iss* (59.2%) was the most frequently detected among the isolates which harboured at least one virulence gene.

For phylogrouping results, a total of 125 *E.coli* isolates were analyzed and their ECOR phylogenetic groups, as determined

Table 3. Distribution of virulence associated genes detected in *E. coli* isolated from chicken

Virulence genes	Number of isolates positive (N)	Percentage (%)
iss	64	51.2
Irp2	14	11.2
iucD	45	36.0
papC	12	9.6
tsh	41	32.8
astA	17	13.6
vat	20	16.0
cva	0	0

Table 4. Phylogenetic groups of *E. coli* isolated from chicken by triplex PCR

Virulence genes	Number of isolates positive (N)	Percentage (%)
A	34	27.2
B1	45	36.0
B2	8	8.8
D	35	28.0

by triplex PCR were as follows: 45 isolates (36.0%) belonged to group B1, 35 isolates (28.0%) belonged to group D, while 34 isolates (27.2%) from group A, to a lesser extent group B2 with only 8 isolates (8.8%). Table 4 shows the phylogenetic grouping based on the PCR results of the *E. coli* isolates tested.

DISCUSSION

Avian isolates are considered as avian pathogenic *E. coli* (APEC) if their PCR results revealed the number of associated virulence genes harboured by the *E. coli* strains (Dziva *et al*., 2010). Several investigations reported that APEC strains expressed certain virulence including adhesion, toxins, iron uptake system and resistance to the host serum. Most of the studies revealed highly diverse virulence genes associated with the APEC strains in poultry. In this study, a high percentage of certain associated virulence genes were detected by multiplex PCR in *E. coli* isolates from chicken including *iss* (51.2%), followed with *iucD*, t*sh, vat, astA, irp2, papC* and *cva* genes. This finding is in agreement with several studies which reported that *iss* gene are most frequently detected in *E. coli* strains isolated from chicken (Skyberg *et al*., 2003; Dissanayake *et al*., 2008; Ewers *et al*., 2009; Yaguchi *et al*., 2010; Kemmet *et al*., 2013; El Hafeez Radwan *et al*., 2014). It has been proposed that *iss* gene can be used as a marker for distinguishing between APEC and commensal strains (Kwon *et al*., 2008). Another study also suggested a wide spread of virulent avian *E. coli* strains due to their resistance to serum complement which is encoded by the *iss* gene (Ewers *et al*., 2009). Presence of the *iss* gene could be used as an indication of the ability of the strains to cause diseases. Most of the virulence genes frequently detected in this study including *iss, tsh* and *iucD* are located in CoIV plasmid, which has been proposed to be associated with *E. coli* virulence (Vandekerchove *et al*., 2005). However, no specific combination of virulence genes which contributes entirely to the pathogenecity of APEC has been reported, until now, as APEC strains may possessed various virulence genes and are not homogenous (Johnson *et al*., 2008).

In this finding, none of the *E. coli* isolates tested harboured the *cva* gene (Table 3), which contradicts previous studies demonstrating the presence of *cva* or *cvi* gene in *E. coli* strains isolated from chicken (Arabi *et al*., 2013; Maturana *et al*., 2011; Roussan *et al*., 2014). A previous study in Malaysia reported that *E. coli* strains isolated from septicemic chickens possessed a high frequency of *cva* gene (Nurul Syuhada *et al*., 2013), and suggested that the *cva* gene is associated with the virulence properties of avian *E. coli*. Low prevalence of *papC* gene identified in avian *E. coli* strains is also in agreement with previous findings reported by Kwon *et al.* (2013) as the *papC* gene is barely detected in both APEC and avian fecal *E. coli* strains. Some studies demonstrated that those virulence genes such as *papC* and

astA genes are likely to be found among avian fecal *E. coli* strains compared to APEC. Thus, these genes were proposed to be not associated with *E. coli* virulence properties (Mohamed *et al*., 2014)

In contrast, the high percentage of *E. coli* isolates tested in this experiment which harbor at least one virulence gene (76.8%) contradicted with other findings reported previously (Kwon *et al*., 2008). Most of the studies showed that more than 40% of the avian *E. coli* strains tested accounted for profiles with more than 4 virulence genes (Kemmet *et al*., 2013). Comparatively, in this study, the percentage is quite low (6.4%). Previous observations indicated that those *E. coli* strains which harbored less than 3 virulence genes are considered as non-pathogenic or commensal *E. coli* (Kwon *et al*., 2008; Oliviera *et al*., 2015). Many APEC strains were classified as commensal strains because they possessed a low number of virulence genes, thus prompting as opportunistic pathogen (Maturana *et al*., 2011).

Even though most of the *E. coli* isolates tested in this study carry less virulence genes, the finding should be taken into consideration as commensal or avirulent *E.coli* strains can serve as a reservoir of virulence genes and have a potential to induce disease when an opportunity arise (Ewers *et al*., 2009; Collingwood *et al*., 2014; Dissanayake *et al*., 2014). The low number of virulence genes in the *E. coli* isolates in this study could be that these isolates harbour other virulence genes that were not screened for, or could be that *E. coli* isolates used belong to the strains that were not highly pathogenic. This fact is in agreement with previous investigations, as other predisposed factors especially environmental factors and other disease factors may induce them to cause diseases leading to colibacillosis (Mbanga *et al*., 2015).

For phylogenetic grouping, APEC strains mainly derived from ECOR phylogroup B2 and occasionally from phylogroup D, while human ExPeC strains mostly belong to group B2 and Group A (Jeong *et al*., 2012; Dou *et al*., 2015).

Most of the *E. coli* isolates used in this study belong to phylogenetic group B1, which accounted for about 36.0% from a total 125 isolates, followed with group D (28.0%), Group A (27.2%) and to a lesser extent group B2 (8.8%). This finding is in agreement with a previous study reported which demonstrated that the majority of avian *E. coli* strains belong to Group B1 (31.8%) and Group A (27.6%) (Kobayashi *et al*., 2011). Other investigations also demonstrated that group A and D were predominant in avian *E. coli* strains in Japan and China (Wang *et al*., 2013; Asai *et al*., 2011; Hiki *et al*., 2014). The high percentage of phylogroup A among *E. coli* isolates tested indicated that the *E. coli* isolates in this study could be non-pathogenic strains, as group A is usually related to commensal *E. coli*. Certain studies suggested that phylogroup A could be responsible for causing extraintestinal infections too (Ewers *et al*., 2009). *E. coli* strains derived from phylogroup A and B1,

which are considered as commensal strains differerent from derivative strains of groups B2 and D as they lack most of the virulence associated genes (Johnson and Russo, 2002). Even though phylogroups A and D are usually considered as commensal and non-pathogenic, the presence of virulence associated genes may be a potential threat in causing diseases when triggered by predisposing conditions (Hiki *et al*., 2014).

Therefore, determination of APEC by identification of phylogenetic groups and the set of virulence associated genes is still debateable because specific phylogenetic groups and profiles of virulence genes associated with APEC strains are presently still unavailable.

CONCLUSION

This study demonstrated that distribution of virulence associated genes among *E. coli* isolates are heterogenous. Phylogenetic grouping of the isolates suggested that *E. coli* isolates tested in this study, derived from commensal strains and certain predisposing factors including environmental and stress factors, may induce them to cause disease. Establishment of recent molecular methods to determine the presence of more virulent genes, other than those in this study, is needed for detailed information on virulent gene profiles of *E. coli* isolates in the country. On the other hand, an *in vivo* chicken infection model can be conducted to determine the correlation of a virulent gene profiles with pathogenecity in chicken.

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