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Virulent Factors of Sorbitol-Negative *Escherichia coli* Isolated from Quail

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Abstract

Colibacillosis in poultry is a disease caused by Avian Pathogenic *Escherichia coli* (APEC) which can affect a significant economic loss in the poultry industry. APEC strains in this study were characterized based on the hemolysin and hemagglutinin production, as well as detection of four essential virulent genes, including the genes of increased serum survival (*iss*), capsule antigen (*kps*), temperature sensitive hemagglutinin (*tsh*) and enterohemolysin (*hly*) gene. In this study, all 21 *Escherichia coli* isolates from quails were negative in the MacConkey Sorbitol test, 38.1% of isolates showed hemolytic and 52.4% hemagglutinin activities. Genotypically, 61.9% of isolates had *iss* gene, *kps* gene for 9.5%, *hly* gene for 9.5%, and 4.8% of isolates had the *tsh* gene. These isolates are pathogenic strains and potentially acts as zoonotic agents.

Key words; *Escherichia coli*, pathogen, quail, sorbitol-negative

Introduction

Avian Pathogenic *Escherichia coli* (APEC) causes colibacillosis in chickens, turkeys as well as other poultry species and gives rise to several forms of bacillosis symptoms such as septicaemia, granuloma, omphalitis, sinusitis, airsacculitis, arthritis/synovitis, peritonitis, pericarditis, perihepatitis, cellulitis, and swollen head syndrome (Kunert-Filho et al., 2015). Colibacillosis in poultry leads to significant economic losses in the livestock industry (Karimi et al., 2011). Avian Pathogenic *Escherichia coli* (APEC) is the leading cause of death in quails compared to other pathogenic bacteria (El-Demerdash et al., 2013).

MacConkey Sorbitol (SMAC) is used as a medium for detection of pathogenic *Escherichia coli* strains (APEC) (Park et al., 2010). Virulence of the sorbitol-negative *Escherichia coli* strain is associated with a number of virulence factors including two cytotoxins encoded as shiga 1 and 2 toxins (stx1 and stx2) (Schmidt et al., 2001). *Escherichia coli* isolates with sorbitol-negative from chickens have the zoonotic risk that threatens human health (Lefebvre et al., 2009).

There are many virulence factors of the APEC strain (Mellata et al., 2003). Phenotypically, the existence of hemolysin and hemagglutinin are the common virulence factors of APEC strain (Radji et al., 2003). Genotypically, some genes are reported as virulent factors in APEC strains, including the adhesin gene, capsular gene and lipopolysaccharide antigen as well as toxins (Musa et al., 2009). Virulence genes in APEC strains isolates from birds have been reported such as adhesins factor, toxins and defense factor (Nakazato et al., 2009). Virulent genes that are important in *Escherichia coli* include increased serum survival (iss) (increased defense against serum) (Paixao et al., 2016; Mitchell et al., 2015), capsular gene (kps) (Johnson et al., 2005), enterohemolysin (hly) and temperature sensitive haemagglutinin (tsh) (Knoble et al., 2011).

In this study, the presence of hemolysin and hemagglutinin were characterized phenotypically, and the occurrence of defence virulence genes against the serum (iss), capsular antigen (kps), enterohemolisin (hly), and temperature sensitive hemagglutinin (TSH) were detected genotypically

in *Escherichia coli* with sorbitol-negative isolates from quails.

Materials and Methods

Escherichia coli Isolates

The study used 21 *Escherichia coli* isolates from quail with sorbitol-negative result at the SMAC test. The SMAC test was carried out according to Koochakzadeh et al. (2014). *Escherichia coli* isolates were cultured in selective Sorbitol MacConkey (SMAC) agar media, incubated at 37°C for 24 hours. *Escherichia coli* colonies with sorbitol-negative grew colourless.

Hemolytic Test

Haemolysin detection was carried out according to Fakruddin et al. (2013). One usa culture of *E. coli* was planted on a sheep's blood agar plate solid medium, then it was incubated at 37ºC for 18-24 hours. Hemolysin was detected by determining the lysis zone around each colony in 5% of the sheep blood agar plates after overnight incubation.

Hemagglutination Test

The hemagglutination test was carried out according to Wibawan et al. (1993). The test used sheep's blood with anticoagulant 0.2 M Sodium Citrate pH 5.2 which was centrifuged and washed twice with 0.15 M NaCl, then made into 2% solution with NaCl. The hemagglutination test was carried out by reacting 20 µl of the bacterial solution which had been determined for its optical density (OD) with a transmission spectrophotometer and λ 620 nm (approximately 10^9 bacteria/ml 0.15 NaCl) with 20 µl erythrocyte solution in a test tube. The test tube was shaken for 30 seconds and the hemagglutination reaction was recorded with the following conditions: ++ strong reaction, + moderate reaction and – no reaction.

Detection of 16SrRNA, *iss, kps, hly and tsh Genes*

Detection of virulence genes was conducted according to Knobl et al. (2003). All isolates were subjected to deoxyribonucleic acid (DNA) extraction and PCR for amplification of the 16SrRNA genes and amplification of vt1 and vt2 genes. DNA extraction was carried out by using DNA extraction kit (Dneasy Qiagen) with the procedure according to the manufacturer's recommendations. Primers designed based on Knobl et al. (2004) were used selective primers for amplifying the 16SrRNA *E. coli* gene and molecular analysis were carried out by using PCR.

Amplification of *E. coli* specific 16SrRNA genes using ECP79F and ECR620R primers was carried out by mixing PCR mix solution (Supermix, Invitrogen, Germany) with 2.5 µl (0.6 µM) of each primer and 2 µl DNA into PCR tube until it reached total volume of 25 µl, then the tube containing the solution is inserted into the thermal cycle tool (Mastercycler, Eppendorf, Germany).

The nucleotide base sequence from the primer and the PCR program are presented in Table 1. The reaction mixture consisted of 2.5 µl of each primary I and primary II, 1 µg DNA and PCR mix to a volume of 25 µl. PCR program for 16SrRNA *E. coli* gene amplification after five minutes of initial denaturation at 94°C, target gene fragment was amplified in 40x cycles. Each cycle with

Table 1. *Polymerase chain reaction* (PCR) of primers for amplification of 16SrRNA, *iss, kps, hly*, and *tsh* genes (Knobl et al., 2004)

No	Gene	Sequence of Primer (5'-3')	Size (bp) of PCR Product
1	16SrRNA	5'-GAAGCTTGCTTCTTTGCT-3' 5'-GAGCCCGGGGATTTCACAT-3'	544 bp
2	*kps*	GCGCATTTGCTGATACTGTTG CATCCAGACGATAAGCATGAGCA	272 bp
3	*hly*	AACAAGGATAAG CAC TGT TCT GGC T ACC ATA TAA GCG GTC ATT CCC GTC A	1.177 bp
4	*iss*	ATCACATAGGATTCTGCCG CAGCGGAGTATAGATGCCA	290 bp
5	*tsh*	ACTATTCTCTGCAGGAAGTC CTTCCGATGTTCTGAACGT	825 bp

a denaturation program for 45 seconds at 94°C, annealing for 45 seconds at 50°C and extension for 1.5 minutes at 72°C (Knobl et al., 2004). Amplification of the *iss, kps,* and *hly* genes were done with thermal cycles and programs according to the reference from Bottero et al. (2004).

PCR products were analysed using electrophoresis with 2% agarose gel (Sigma), and buffer (TAE) (0.04 M Tris; 0.001 M EDTA; pH 7.8). A total of 10 µl of PCR product was mixed with ± 3 µl of loading buffer, then electrophoresis was carried out using 2% agarose gel at 100 V for 30 minutes. After electrophoresis, the DNA bands on the agar were stained with Sybr save staining solution and visualized using UV transilluminator.

Results and Discussions

APEC is a pathogenic strain as a cause of colibacillosis in poultry, including quails. The detection of APEC strains was phenotypically conducted with Sorbitol MacConkey (SMAC) selective media (Arslan and Ozdemir, 2013). The results of the identification of APEC strains with SMAC media are presented in Figure 1 and Table 2. Twenty-one *Escherichia coli* isolates were phenotypically identified as APEC strains. The isolates grew white colonies in the SMAC media. Sorbitol MacConkey (SMAC) is a selective media to isolate APEC strains (Müller and Ehlers, 2005). APEC strain grows transparent colorless colonies in the SMAC media (Koochakzadeh et al., 2014).

Identification of Avian Pathogenic *Escherichia coli* (APEC) with SMAC media has been conducted by Kim et al. (2005). According to Lefebvre et al. (2009), *Escherichia coli* w sorbitol-negative originating from poultry is important zoonotic strain. APEC strains ha several virulence factors, both phenotypica and genotypically. In this study, the presence hemolysin and hemagglutinin indicated that APEC strains are a pathogen. Hemolysin a hemagglutinin are reported as the virulent fact in *Escherichia coli* (Kausar et al., 2009).

Detection of hemolysin in *Escherichia c* was conducted using blood agar media, as sho in Figure 2. In this study, 38.1% of isolates h hemolysin (hemolytic strain). Roy et al. (2006) reported the prevalence of hemolytic strains in *Escherichia coli* isolates from quail was 45.2%. The hemolytic prevalence of broiler origin isolates was reported to be higher than that of quail isolates. Al-Arfaj et al. (2016), McNamee et al. (1998), Zahid et al. (2016), Radwan et al. (2014) and Fakruddin et al. (2013) reported 92.9%, 86.4%, 53.3%, 41% and 44.6% of hemolytic isolates respectively. Some researchers report a smaller prevalence of hemolytic strains of poultry origin isolates compared to the results of this study. Shankar et al. (2010), Catana and Herman, (2010) and Fodor et al. (2010) reported hemolytic strains of 3.9%, 5%, and 19%, respectively.

Hemolysin is one of the virulence factors possessed by *Escherichia coli* and is associated with pathogenesis (Kukanur et al., 2015) and is responsible for the severity of infection (Johnson, 1991). Hemolysin functions to damage erythrocytes and increase pathogenicity by destroying phagocytes and epithelial cells (Naveen and Mathai, 2005), and serves to enhance the ability of *Escherichia coli* to survive against phagocytosis in the bloodstream (Welch, et al., 1995).

Figure 1. The growth of *Escherichia coli* on the Sorbitol MacConkey media with the white colonies indicated the negative Sorbitol strain.

Hemolytic Escherichia coli causes disease more often than non-hemolytic (Grover et al., 2013), and causes the condition to continue more seriously (Mittal et al., 2014). Phenotypically, the presence of hemolysin can be detected by the formation of a zone of hemolysis around colonies that grow on sheep blood agar (Beutin et al.,

Figure 1. The growth of Escherichia coli on Sorbitol MacConkey media: Escherichia coli with negative sorbitol, white colored colony

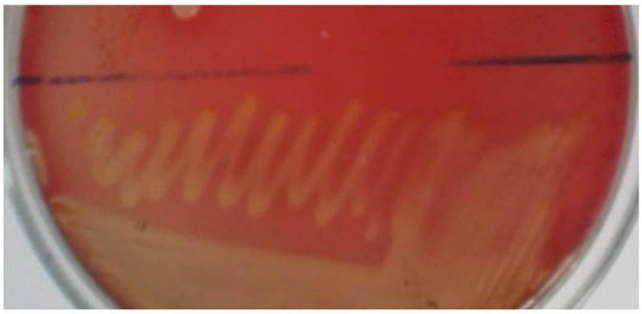

Figure 2. Hemolysis of *Escherichia coli* on the sheep blood agar

1989), this method is fast and straightforward to determine the virulence of Escherichia coli (Moon et al., 2006).

Hemolysin released by bacteria will cause lysis of red blood cells (Coote, 1996) and hemolysin is the only protein that lyses red blood cells (Herlax et al., 2010). Hemolytic activity indicates that the APEC strains are virulent (Hassan and Bakeet, 2014). It has been demonstrated in vivo that *Escherichia coli* hemolytic strains converted to non-hemolytic strains will reduce virulence in experimental mice (Welch et al., 1981).

In this study, 52.4% of isolates had hemagglutinin, which was characterized by the ability of bacteria to clot sheep red blood cells in the hemagglutination test (Figure 3). The occurrence of blood clots indicates the presence of hemagglutinin due to the bond between *Escherichia coli* and erythrocytes (Wibawan et al., 1993). The hemagglutination test is a test of the existence of hemagglutinin of *Escherichia coli.* It is an essential virulent factor in APEC strains. Hemagglutinin is an adhesin serving as a sticking factor for *Escherichia coli* in cells and tissues and

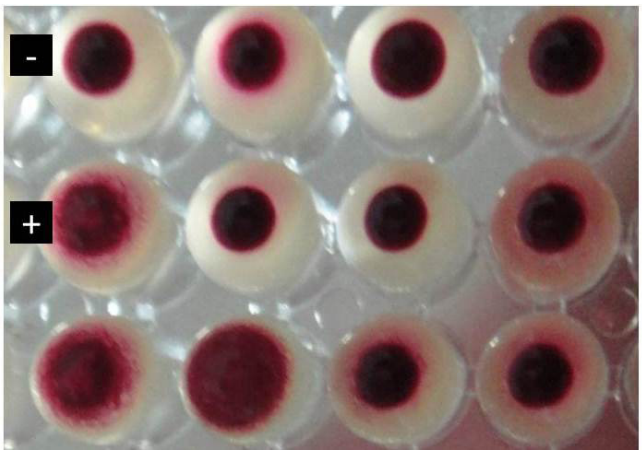

Figure 3. Hemagglutinin reaction of Escherichia coli

agglomerates erythrocytes (Lutwyche et al., 1994; Abrar et al. 2009). Hemagglutinin is related to the ability of bacteria to attach to the surface of epithelial cells in the infection process (Edwards et al., 2000), and plays a role in agglomerating red blood cells (Maheswari et al., 2013; Raksha et al., 2003).

Some research results reported that the prevalence of APEC strains that have hemagglutinin is lower than the results of this study. Sharada et al. (2010), Hamad et al. (2012) and Al-Saiedi et al. (2014) reported *Escherichia coli* isolates from poultry had hemagglutinin at 20%, 18.2%, and 3.70% respectively. Conversely, some researchers report higher than the results of this study as reported by Radwan et al. (2014) and Oh, et al. (2011) with the result 100% and 82.3%, respectively.

Genotypically, virulence factors in APEC strains can be grouped as adhesin genes, iron acquisition, hemolysis, defense against bactericidal, and toxin production (Dziva and Stevens, 2008; Johnson and Nolan, 2009). APEC strains have many virulence factor genes related to pathogenicity (Foley et al., 2000), but not all virulence genes in *Escherichia coli* are found in the same isolates (Maturana et al., 2011; Olsen et al., 2012), and no isolate had only a single virulence factor (Foley et al., 2000).

Virulent genes that are important in *Escherichia coli* include the *iss, kps* and *tsh* genes (Drugdova and Kmet, 2013), as well as *hly* genes (Morales et al., 2004; Lefebvre et al. 2009). In this study, the prevalence of *iss, kps, tsh* and *hly* genes were 61.9%, 9.5%, 0% and 9.5% respectively (Table 3).

This study revealed that the prevalence of *iss* genes is 61.9%. The *iss* gene is a virulent gene that is important in *Escherichia coli* (Dissanayake et al., 2014; Paixao et al., 2016), and it is the gene which is most commonly found in APEC strains (Kwon et al., 2008). The prevalence of the *iss* gene in this study is similar to the results of studies reported by Badouei et al. (2015) and Al-Arfaj et al. (2016), in which the prevalence of *iss* genes in APEC strains of broiler isolates were 64.3% and 64.29% respectively.

Yaguchi et al. (2007), Cunha et al. (2014), Badouei et al. (2015) and Abd El Tawab et al.

(2016) reported higher prevalence of *iss* genes than the results of this study, which were 97.6%, 93%, 90.3% and 86.6% respectively. The prevalence of the *iss* gene is higher in APEC strains originating from chickens with colibacillosis (Ewers et al., 2014; Dissanayake et al., 2014) compared to the prevalence of *iss* genes in APEC strains isolates from healthy chicken faeces (Rodriguez-Siek et al., 2005). Mohamed et al. (2014) did not find the *iss* gene for APEC strains from healthy chicken faeces.

Gen *iss* plays a role in increasing survival of the bacteria in host serum fluids (Kwon et al., 2008; Pfaff-McDonough et al., 2000), increasing ability to resist bactericidal effects (Johnson et al. 2002), and causing severity colibacillosis in broiler chickens (Kwon et al., 2008).

Table 2. The presence of haemolysin and hemagglutinin, *kps*, *hly, iss* and *tsh* genes of the APEC isolates

No	Sample code	SMAC	Hem olysin	Hem aglutinin	kps	hly	iss	tsh
1	2	-	-	-	-	-	-	-
2	6	-	+	+	-	-	-	-
3	12	-	-	+	+	-	-	-
4	16	-	+	+	-	-	+	-
5	18	-	-	+	-	-	+	-
6	19	-	-	+	-	-	+	-
7	20	-	+	+	+	+	-	-
8	P2	-	+	+	-	-	+	-
9	P4	-	-	-	-	-	+	-
10	P5	-	+	+	-	-	+	-
11	P6	-	-	-	-	-	+	-
12	P10	-	+	+	-	-	-	-
13	P12	-	-	-	-	-	+	-
14	P15	-	-	-	-	-	+	-
15	P17	-	-	-	-	+	+	-
16	P18	-	-	-	-	-	-	-
17	P19	-	-	-	-	-	-	-
18	P5.3	-	-	-	-	-	+	+
19	P11.1	-	+	+	-	-	+	-
20	P17.1	-	-	-	-	-	+	-
21	P16.2	-	+	+	-	-	-	-
	%	100	38,1	52,4	9,5	9,5	61,9	4,8

Table 3. Distribution of virulence factor phenotypically and genotypically of the APEC isolates

aglu = hemagglutinin
iss = *increased serum survival* gene
tsh = *temperature sensitive hemagglutinin* gene
hemo= hemolysin
hly = hemolysin gene
kps = capsular gene

The polysaccharide capsule is a bacterial surface antigen and acts as a virulence factor of the APEC strain (Lynne et al., 2007). Polysaccharide capsule is codified as the *kps* gene (Johnson et al., 2005). Polysaccharide capsule plays a role in the adhesion process with host tissue cells in the early stages of infection (Stathopoulos et al., 1999) and acts as a factor in bacterial survival in the host serum (Mellata et al., 2003) by avoiding host innate immune responses (Huja et al., 2015; Dziva et al., 2013).

Pourbakhsh et al. (1997) reported that bacteria having polysaccharide capsules are resistant to the bactericidal effects contained in the host serum. The ability of bacteria to survive and grow in serum and tissue becomes an important virulence determinant of APEC strains and plays a role in the pathogenesis of colibacillosis (Gao et al., 2012). Escherichia coli which has the kps gene is a pathogenic strain (Pfaff-McDonough et al., 2000; Monroy et al., 2005). In this study 9.5% of isolates had the kps gene. The APEC strain isolates from broilers reported by Rocha et al., (2008) is 18%, Vidotto et al. (1999) is 11.1%. However, the result of this study is higher than the results reported by Mbanga and Nyararai (2015) for about 2,2%.

Temperature sensitive haemagglutinin is coded as *tsh* genes (Provence and Curtiss, 1994). *Escherichia coli* isolates that have the *tsh* gene are pathogenic strains (Lefebvre et al., 2009). The *tsh* gene plays a role in the adhesion process (Paixao et al., 2016) at the beginning of colonization of infection (Delicato et al., 2002; Tivendale et al., 2004), causes proteolytics (Kostakioti and Stathopoulos 2004), and plays a role in the process red blood cell clotting (Ghunaim et al., 2014; Huja et al., 2015). The *tsh* gene plays a role in the severity of colibacillosis in poultry (Paixao et al., 2016), and often causes death (Dozois et al., 2000).

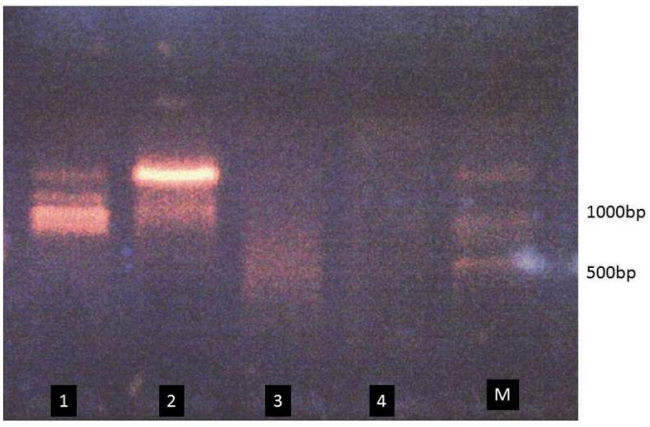

Figure 4. The electrophoresis of PCR product. 1. *tsh* gene (825 bp), 2. *hly* gene (1.17 bp), 3 and 4. Negative of *kps* and *iss* genes. M. Marker.

In this study, 1 isolate (4.8%) had the *tsh* gene. The results of this study are similar to the results of a study by de Campos et al. (2005) in which the prevalence of genes in the APEC system from broilers was reported at 6%. Some researchers report various prevalence of *tsh* genes in APEC strains from poultry. Zhao et al. (2005) reported that 84% of APEC strains from broilers had the *tsh* gene; 56% of APEC strains from colibacillosis in meat and laying hens have the *tsh* gene (Ewers et al., 2004); 55.7% of APEC strains from turkeys with *tsh* genes were reported by Cunha et al. (2014) and 46% in study by Abd El Tawab et al. (2016).

Genes of *hly* play a role in adaptation to improve survival and increase virulence (Shtylla et al., 2012). The presence of the *hly* gene is associated with the presence of haemolysin and the ability to lyse red blood cells. Hemolysin is one of virulence factors that helps in the pathogenesis of *Escherichia coli* (Reingold et al., 1999). In this study, 2 isolates from 21 (9.5%) were detected as having *hly* genes. Hamza et al., (2016), Jamshidi et al., (2016), Morales et al. (2004) and (Al-Arfaj, 2016) reported the prevalence of APEC *hly* strain genes of 8.3%, 1.28%, 25.0% and 92.9%, respectively. In this study, only 12.5% of isolates having haemolysin also had *hly* genes. According to Morales et al. (2004) and Hamza et al. (2016), the existence of haemolysin is not always the same phenotypically and genotypically.

This study revealed that 2 isolates from 21 (9.5%) did not have the six virulence factors detected, 7 isolates (33.3%) had only 1 virulence factor, 7 isolates (33.3%) had 2 virulence factors, 4 isolates (19.0%) had 3 virulence factors and 1 (4.8%) isolate had 4 virulence factors. Isolates that were not detected of having virulence factors did not necessarily free from virulence factors. It is possible that those isolates have other virulent factors that were not tested. The presence of several virulence factors will increase virulence, and virulence in *Escherichia coli* is multi-factorial (Rocha et al. 2008). The synergy of activities of

the factors increases pathogenicity and overcomes host defense (Samy et al., 2013).

Conclusion

According to the phenotypically and genotypically determination, APEC strains from quails have several virulence factors. The virulence factors are multi-factorial to increase virulence. APEC strains from quail are pathogenic strains and have potentially transmit to other animals and humans.

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