

## Virulence Characteristic of Avian Pathogenic *Escherichia coli* (APEC) Isolates

### *Karakteristik Virulensi Isolat Avian Pathogenic Escherichia coli (APEC)*

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#### Abstrak

*Avian pathogenic Escherichia coli* (APEC) merupakan penyebab kolibasilosis pada unggas, salah satu penyakit pernapasan yang menyebabkan permasalahan yang serius di industri perunggasan. APEC dapat mengakibatkan tingginya jumlah kematian dan angka afkir, penurunan produksi, dan tingginya biaya pengobatan. Manifestasi kolibasilosis yang biasa terjadi adalah *airsacculitis*, perihepatitis, dan perikarditis. Serotipe APEC yang banyak diidentifikasi di lapangan adalah O1K1, O2K1, dan O78K80. *Embryo lethality assay* (ELA) adalah salah satu metode yang dapat digunakan untuk mengetahui virulensi serotipe APEC. Tujuan dari penelitian ini adalah untuk mengetahui karakteristik virulensi isolat APEC berbagai serotipe. Lima isolat APEC serotipe O1K1, O2K1, O78K80, O157H7, dan *unknown serotype* digunakan untuk pengujian virulensi dengan menginokulasikan bakteri ke dalam cairan alantois telur ayam berembrio *specific pathogen free* (TAB SPF) umur 12 hari. Masing-masing serotipe dibutuhkan telur sebanyak 10 butir, dengan dosis bakteri 100-500 CFU/ 0.1 ml. *Candling* dilakukan setiap hari, sampai umur embrio 18 hari, untuk menentukan jumlah kematian dan perubahan lesi patologi. Setelah diamati selama 6 hari, persentase TAB yang mati setelah diinokulasi dengan APEC serotipe O1K1, O2K1, *unknown serotype* adalah 100% (10/10), serotipe O78K80 90% (9/10), dan serotipe O157H7 70% (70%). Seluruh ayam yang mati mengalami lesi patologi adanya hemoragi daerah kranial dan ekstremitas. Seluruh isolat *E. coli* dalam penelitian ini memiliki virulensi tinggi.

**Kata Kunci:** avian pathogenic *E. coli* (APEC); serotipe *E. coli*; virulensi; embryo lethality assay

#### Abstract

*Avian pathogenic Escherichia coli* (APEC) is a cause of colibacillosis in poultry, one of the respiratory disease that causes serious problems in the poultry industry. The APEC can cause high mortality and culling, decreased production, and high costs of treatment. Manifestations of colibacillosis are *airsacculitis*, perihepatitis, and pericarditis. The APEC serotypes that are widely identified in the field are O1K1, O2K1, and O78K80. *Embryo lethality assay* (ELA) is a method for to determine the virulence of APEC serotypes. The aim of this study is to determine the virulence characteristic of APEC isolates. Five APEC serotypes O1K1, O2K1, O78K80, O157H7, and *unknown serotype* were used for ELA method by inoculated *E. coli* into chorioallantoic of specific

pathogen free 12-days old embryos. Each group of 10 embryos, inoculated *E. coli* dose of 100-500 CFU/ 0.1 ml. Candling was carried out for 6 days (18-days old embryo) to determine the mortality and pathological lesions. The percentage of embryo mortality post-inoculated with APEC O1K1, O2K1, unknown serotypes were 100% (10/10), O78K80 serotype was 90% (9/10), and O157H7 serotype was 70% (7/10). Lesions of all embryos were cranial and extremity hemorrhage. In this study, *E. coli* isolates had high virulence.

**Key words:** avian pathogenic *E. coli* (APEC); *E. coli* serotypes; virulence; embryo lethality assay

## Introduction

Respiratory infection is one of the main problems in the poultry industry. The infection causes economic loss due to the increase of mortality and culling, decrease of carcass quality, production, and high costs of treatment. Colibacillosis is one of the respiratory infections caused by APEC. The APEC is the extraintestinal group of *E. coli* which causes various internal organ lesions/ septicemia such as airsacculitis, perihepatitis, pericarditis, cellulitis, egg peritonitis, salpingitis, coligranuloma, omphalitis, and osteomyelitis/ arthritis (Chansiripornchai *et al.*, 2011; Ghunaim *et al.*, 2014; Gross, 1991; Kemmett *et al.*, 2013; Roseliza *et al.*, 2016). This systemic infection occurs when a large number of pathogenic *E. coli* into blood vessels through the respiratory or digestive tract (Chansiripornchai, 2009).

Prevention of APEC infections has been carried out by vaccination of other respiratory agents and *E. coli* itself, reducing stress in chickens, predisposition factors, and treatment with antibiotics (Chansiripornchai, 2009; Cortes *et al.*, 2010; Ghunaim *et al.*, 2014; Wibowo and Amanu, 2009). The increase of antibiotics resistance to *E. coli* and residues in meat should be the concern (Gregersen *et al.*, 2010). Whereas *E. coli* vaccination provides a better result if the vaccine serotypes are homolog with field strain (Ahmed *et al.*, 2015; Dho-Moulin and Fairbrother, 1999; La Ragione *et al.*, 2013). In addition, there is no diagnostic tool to differentiate the *E. coli* isolates from primary (highly virulent), secondary (moderately secondary), or non-pathogenic (avirulent) isolates (Wooley *et al.*, 2000; Oh *et al.*, 2012).

Effective and reliable methods for bacterial virulence testing require a research tool (Andersson *et al.*, 2015). Embryo lethality assay (ELA) can be used to differentiate between virulent and

avirulent *E. coli* isolates (Nolan *et al.*, 1992). The ELA method is a sensitive method to determine *E. coli* virulence in diagnostic laboratories, by the percentage of embryo mortality after inoculation (Oh *et al.*, 2012).

## Materials and Methods

### *Escherichia coli* Isolates

The *E. coli* serotypes for inoculated were O1K1, O2K1, O78K80, O157H7, and unknown serotype (collection of Veterinary Medicine Faculty of Gadjah Mada University). All serotypes were reisolated on media eosin methylene blue (EMB), Gram staining, and biochemical tests (Triple Sugar Iron Agar/ TSIA, urea, semisolid, indole, methyl red (MR), Voges-Proskauer (VP), catalase, citrate, sorbitol, glucose, lactose, sucrose, and mannitol). The bacteria were confirmed as *E. coli* then used as an inoculum.

### Inoculum

The inoculum was an isolate that confirmed as *E. coli* on EMB media, Gram staining, and biochemical tests. The *E. coli* then grown on brain heart infusion (BHI) media at 37°C for 24 hours, then washed with PBS solution, and compared with standard McFarland 0.5. The number of bacteria inoculated is 100-500 CFU/ 0.1 ml (Nolan *et al.*, 1992; Powell & Finkelstein, 1966; Wooley *et al.*, 2000). The determination of the bacteria number was by the total plate count (TPC) method on nutrient agar (NA), with sample dilutions had been standardized with McFarland 0.5.

### Embryo Lethality Assay (ELA)

Fifty-five of specific pathogen free 12-days old embryos were divided into 5 groups based on *E. coli* serotypes (O1K1, O2K1, O78K80, O157H7, and unknown serotype). Each group of 10 embryos treatment and an embryo control was inoculated

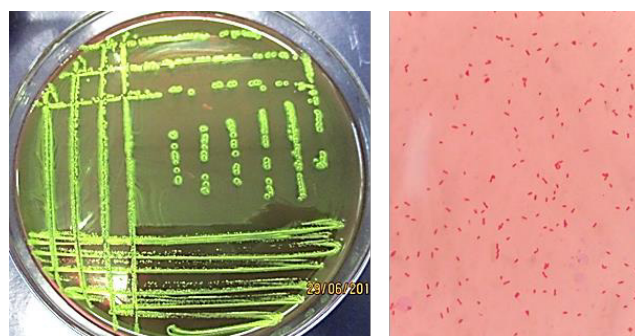
with PBS. Each treatment group was inoculated with each *E. coli* serotype in chorioallantoic. Candling is carried out for 6 days (18-days old embryo) to determined the mortality. Mortality was recorded and observed for pathological lesions (Gibbs *et al.*, 2003; Nolan *et al.*, 1992; Oh *et al.*, 2012; Powell & Finkelstein, 1966; Wooley *et al.*, 2000). There were virulent characteristic as described by previous study (Wooley *et al.*, 2000), where the mortality of embryos < 10% is avirulent, 10-29% is moderate virulence, and > 29% is high virulence. Reisolation of chorioallantoic was on EMB media, as confirmation of the cause of death was *E. coli*.

## Results and Discussion

### *Escherichia coli* Isolates

The *E. coli* serotypes O1K1, O2K1, O78K80, O157H7, and unknown serotype on EMB showed typically colony of metallic sheen (Figure 1a). The Gram staining showed a small rod Gram-negative (Figure 1b). This result is according to Swayne *et al.* (2013), Gordon and Jordan's (1982) where the growth of *E. coli* in EMB is metallic sheen, compared with the other *Escherichia* species. The cell morphology of bacterium is a small rod, Gram-negative, and does not have spores.

The results of biochemical tests, serotypes O1K1, O2K1, O78K80, O157H7, and unknown



**Figure 1.** Characteristics of *E. coli* isolate. (A) The morphology of the *E. coli* on the EMB media was metallic green, (B) The *E. coli* cells were small rods, Gram-negative (red staining).

serotype on TSIA media could ferment all carbohydrates (glucose, lactose, sucrose) and have CO<sub>2</sub> gas. Urea, VP, and citrate were negative results. The bacterial positive on semisolid media, because *E. coli* have flagella. All isolates could fermented carbohydrates (sorbitol, glucose, lactose, sucrose, and mannitol), changed the color of the media to yellow, except unknown serotype which cannot ferment sucrose. All biochemical results according to Abbott *et al.* (2003) and Nataro *et al.* (2011). The biochemical results are presented in Table 1

### Embryo Lethality Assay (ELA)

The number of APEC serotypes O1K1, O2K1, O78K80, O157H7, and unknown

**Table 1.** The biochemist results of *E. coli* serotypes

Biochemist	<i>E. coli</i> serotypes				
	O1K1	O2K1	O78K80	O157H7	Unknown serotype
TSIA	S: yellow B: yellow	S: yellow B: yellow	S: yellow B: yellow	S: yellow B: yellow	S: yellow B: yellow
Urea	-	-	-	-	-
Semi solid	+	+	+	+	+
Indol	+	+	+	+	+
MR	+	+	+	+	+
VP	-	-	-	-	-
Catalase	+	+	+	+	+
Citrate	-	-	-	-	-
Sorbitol	+	+	+	+	+
Glucose	+	+	+	+	+
Lactose	+	+	+	+	+
Sucrose	+	+	+	+	-
Mannitol	+	+	+	+	+

S (slant), B (butt), + (positive result/ ferment carbohydrate), - (negative result/ couldn't ferment carbohydrate)

serotype as much as 100-500 CFU/ 0,1 ml were determined by the TPC method, then inoculated on chorioallantoic of 12-days old embryos, each 10 embryos/ serotype. After 6 days (18-days old), the result of *E. coli* serotypes O1K1, O2K1, and unknown serotype were the highest percentage of embryo mortality (100%) than the other APEC serotypes. The O78K80 serotype has 90% of mortality, and the O157H7 serotype has 70% (Table 2). The chickens in the control group did not have mortality. Virulence characteristics based on the percentage of embryo mortality according to Wooley *et al.* (2000), all the *E. coli* serotypes O1K1, O2K1, O78K80, O157H7, and unknown serotype were highly virulent. The high virulence of serotypes O1K1, O2K1, and O78K80 were in line with Wahyuardani *et al.* (2014) with a case of broiler colibacillosis in Yogyakarta and Bogor, which were O1K1, O2K1, and O78K80 all of the 14 samples of *E. coli* isolates.

The 10 embryos used in each isolate to determine the *E. coli* virulence by previous research (Nabbut and Khatib, 1978). The percentage of mortality post-inoculated was significantly high, medium, or avirulent of APEC isolates (Wooley *et al.*, 2000). The different mortality shows the ability of each isolate to be able to kill the embryo (Gibbs *et al.*, 2003).

Although O1, O2, and O78 are the main *E. coli* serotypes that cause colibacillosis, there were many studied for other serotypes that cause colibacillosis, including O25, O125, O153 serotypes. This is proved that *E. coli* serotype is not an important factor for the pathogenesis of colibacillosis in chickens (Ozaki and Murase, 2009). The *E. coli* O157 serotype is one of the pathogenic serotypes in chickens experimentally. The *E. coli* O157 serotype is not only pathogens that infect poultry, but also in humans. Several

studied have shown that poultry already has and persistently infected with *E. coli* O157 serotype (El-Sawah *et al.*, 2018). The O157 serotype has also been reported isolated from pigeons, have lesions and histopathology that characterizes APEC infections (Dutta *et al.*, 2013).

**Embryonic Lesions and Reisolation of Bacteria**

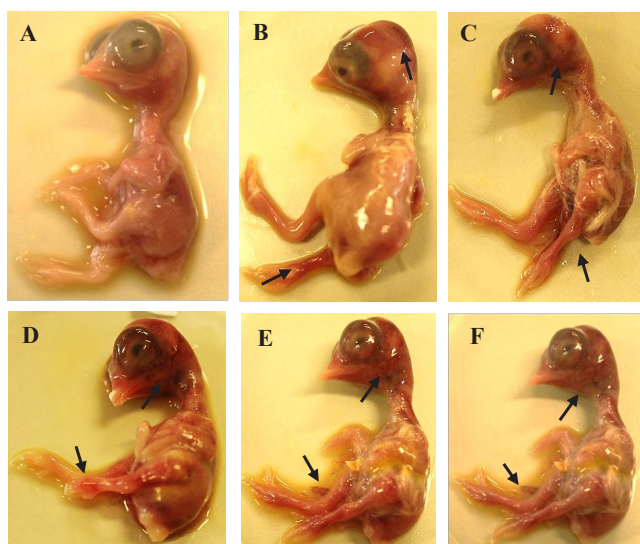
Lesions in dead embryos were cranial, skin, and extremity hemorrhages (Figure 2). These results according to Wooley *et al.* (2000), skin and cranial hemorrhages to encephalomalacia in embryos inoculated with virulent isolates. The presence of hemorrhage in the embryo is due to the massive growth and multiplication of bacteria in the tissues, the production and release of hemolytic factors, and other toxin factors (Nabbut and Khatib, 1978).

The *iss* gene is a virulence factor and strongly correlates with virulent *E. coli* isolates (Nolan *et al.*, 1992). The *iss* gene of isolates can predict the percentage of embryo mortality. The *iss* genes, resistant complement, and CoIV production are strongly associated with virulence potential by chicken embryo lethality method. The chicken embryo can be used to distinguish APEC from commensal *E. coli* (Gibbs *et al.*, 2003).

According to Wooley *et al.* (2000) control of colibacillosis is difficult because the lack of a diagnostic tool to determine the virulence of isolates. The results of this study are easy to do to determine virulence among isolates, because of the significant number of mortality and percentages (high ability to kill embryos). Most embryos would died 3-4 days post-inoculation, the most mortality usually 2 days post-inoculation (Gibbs *et al.*, 2003; Nabbut and Khatib, 1978; Nolan *et al.*, 1992; Powell and Finkelstein, 1966).

**Table 2.** The number and percentage of embryo mortality post-inoculated with APEC serotypes

<i>E. coli</i> serotypes	Embryo mortality post-inoculated/ day						Total	Percentage of mortality
	1	2	3	4	5	6		
O1K1	6	4	0	0	0	0	10/10	100%
O2K1	2	2	2	0	0	4	10/10	100%
O78K80	2	6	1	0	0	0	9/10	90%
O157H7	2	0	4	0	1	0	7/10	70%
Unknown serotype	6	4	0	0	0	0	10/10	100%



**Figure 2.** Lesions of embryo (cranial, skin, and extremity hemorrhage). (A) control, (B) inoculated with O1K1 serotype, (C) inoculated with O2K1 serotype, (D) inoculated with O78K80 serotype, (E) inoculated with O157H7 serotype, (F) inoculated with unknown serotype.

### Conclusion

The virulence characteristics of *E. coli* serotypes O1K1, O2K1, O78K80, O157H7, and unknown serotype was highly virulence.

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