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Characterisation of *E. coli* Virulence Factors Causing Diarrhea in *Macaca fascicularis* at IPB University Captivity, Bogor, Jawa Barat, Indonesia

(Karakteristik Faktor Virulensi *E. coli* Penyebab Diare pada Penangkaran *Macaca fascicularis* di IPB University, Bogor, Jawa Barat, Indonesia)

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ABSTRAK

Diare pada *Macaca fascicularis* sangat mengganggu proses penangkaran, diare mengurangi kualitas hidup kera, *E. coli* sering dilaporkan sebagai penyebab utama diare pada beberapa primata non-manusia. Tujuan dari penelitian ini adalah untuk mendeteksi faktor virulensi seperti hemolisin, pola hemaglutinin, dan hidrofobisitas permukaan sel. Penelitian ini menggunakan 30 isolat *E. coli* dari koleksi pusat penelitian primata IPB University yang diisolasi dari kera ekor panjang yang menderita diare. Faktor virulensi seperti hemolisin, hemaglutinasi, dan agregasi garam dideteksi sesuai protokol standar. Sebanyak 30 isolat *M. fascicularis* yang menderita diare diuji kemampuannya menyebabkan lisis pada eritrosit, di antara 24 (80%) sampel *E. coli* menunjukkan α -hemolisin pada agar darah dan 2 (6,67%) sampel menunjukkan γ-hemolisis. Sebanyak 21 (70%) sampel *E. coli* mampu menghemaglutinasi eritrosit sapi dan 27 (90%) sampel mampu menghemaglutinasi eritrosit kelinci. Sebanyak 30 sampel *E. coli* tidak mampu menghemaglutinasi eritrosit ayam, kucing, anjing, dan manusia. Sebanyak 2 (6,67%) sampel menunjukkan hasil positif pada uji agregasi garam di antara 30 sampel *E. coli* dari *M. fascicularis* dan sisanya 28 (93,33%) sampel tidak menunjukkan hasil positif pada uji agregasi garam. Kesimpulan penelitian adalah Sebanyak 30 isolat *E. coli* menunjukkan variasi faktor virulensi yang berperan dalam mekanisme diare pada *Macaca fascicularis*. Terdapat 24 isolat (80%) yang menunjukkan α-hemolisin, 2 isolat (6,67%) menunjukkan γ-hemolisin, dan 4 isolat lainnya (13,33%) tidak menunjukkan aktivitas hemolisis. Selain itu, 29 isolat (96,7%) menunjukkan hemaglutinasi terhadap eritrosit sapi dan/atau kelinci, sedangkan 2 isolat (6,7%) bersifat hidrofobik berdasarkan uji salt aggregation.

Kata Kunci: Diare, *E.coli*, Faktor Virulensi, *Macaca fascicularis*

INTRODUCTION

Escherichia coli, is a rod-shaped bacteria in the gram-negative group which is member of the *Enterobacteriaceae* family, which was first isolated and characterized by Theodor Escherich in 1885, a few hours after the birth, *E. coli* colonized and inhabited the baby's digestive tract. Generally, *E. coli* is a bacteria that has commensal benefits in balancing the digestive environment (Cobo-Simon and Ochman, 2023). However, commensal *E. coli* can change to caused disease in humans and animals, when the gastrointestinal barrier is damaged or the host in immunocompromise condition. Some *E. coli* also mediated various intestinal and extraintestinal diseases in humans and animals worldwide (Ramos et al. 2020).

Diarrhea in *M. fascicularis* is very disruptive in the captive breeding process, diarrhea reduced the quality of life of macaques, caused death in infant-juvenile, malnutrition, and allows infection with other diseases. *Escherichia coli* is often reported as the main cause of diarrhea in several non-human primates (Abdallah et al 2020), in rhesus monkeys (Wu et al. 2024), *P. anubis* (Waititu et al. 2022), *M. fascicularis* (loe et al. 2021), however, the cause of diarrhea in *E. coli* is still unclear. It is important to detected virulence factors in order to determined the disease's pathogenicity and developed a therapeutic plan to overcame the disease (Pabkin et al. 2021)

The novelty of this study lies in the identification and characterization of *E. coli* virulence factors specifically circulating in captive *Macaca fascicularis*, which has not been comprehensively reported in previous research. This study provides new insights into the pathogenic potential of *E. coli* in this species and contributes essential baseline data for developing targeted diagnostic and therapeutic strategies in captive primate management.

MATERIALS AND METHODS

Ethical approval and informed consent

The study was conducted according to the princi ples of the animal welfare. The protocol was approved by the animal ethics committee, School of Veterinary Medicine and Biomedical Science, IPB University. The ethical approval number is: 260/KEH/SKE/X/2024.

Research design

This study used 30 *E. coli* isolates from the primate research center IPB University collection which was isolated from long-tailed macaques suffered from diarrhea by Loe *et al* (2021). *Escherichia coli* isolates were re-identified again using EMB agar media, gram staining, and observation with a microscope. Confirmed *E. coli* isolates were cultured on TSA media.

Haemolisin

The cytolytic protein toxin secreted by hemolytic *E. coli* isolates is alpha-hemolysin, beta-hemolysin and gamma-hemolysin. Haemolysin was detected by inoculating the strains on 5% sheep blood agar plates and kept for overnight incubation at 37 ℃. The test is hemolytic

when a zone of lysis is seen around each colony on blood agar plates.

Hemaglutination pattern

Hemagglutination assay used 30 isolates of *E. coli* cultured in BHI at 37 ℃ for 24 hours, Fimbriated *E. coli* produces hemagglutination, which can be detected by showing the presence of clumping of erythrocytes. The blood is washed three times with normal saline, and a 1% erythrocyte suspension is then prepared with phosphate buffer saline (pH 7.4). *E. coli* grown on BHI is inoculated into 5 ml of phosphate buffer saline pH 7.4. The procedure is carried out on a microplate. Each of 40 µl of bacterial suspension was mixed with 40 µl of human, cow, rabbit, chicken, cat, and dog blood. The slide is then gently shaken, and the hemagglutination reaction is recorded.

Salt Aggregation Test (SAT)

The hydrophobic characteristic of the bacterial strains was determined according to the method reported by Jonsson P. and Wadstrom T., (1984). Samples were grown in 10 mL of BHI broth at 37 °C for 18 h. Bacterial cells were harvested by centrifugation (3000 g for 15 min), washed twice with phosphate-buffered saline (PBS) pH 7, and suspended in PBS at a concentration of 10^7 cells/mL. Bacterial cell suspensions (25 µL) were mixed with equal volumes of ammonium sulfate of various molarities (0.2,0.4,0.5,0.8,16 and 3.6 mol/L) on microscopic glass slides. The lowest concentration of ammonium sulfate giving a visible aggregation was scored as the SAT hydrophobicity value.

RESULT AND DISCUSSION

Total of 30 isolates of *M. fascicularis* suffered from diarrhea were tested for their ability to cause lysis in erythrocytes, among 24 (80%) *E. coli* samples showed α-hemolysin on blood agar and 2 (6.67%) samples showed γ -hemolysis (Figure 1). Total of 21 (70%) *E. coli* samples could hemagglutinated bovine erythrocytes and 27 (90%) samples could hemagglutinated rabbit erythrocytes (Figure 2). Total of 30 *E. coli* samples were unable to hemagglutinated chicken, cat, dog, and human erythrocytes. The distribution of hemagglutinin patterns tested on *E. coli* isolates in this study is tabulated in Table 1.

Total of 2 (6.67) samples were positive for salt aggregation test among 30 samples of *E. coli* from *M. fascicularis* and rest 28 (93.33%) do not showed positive result in salt aggregation test (Figure 3)**.** The distribution of the virulence factor tested among *E. coli* isolates in this study are tabulated in Table 2.

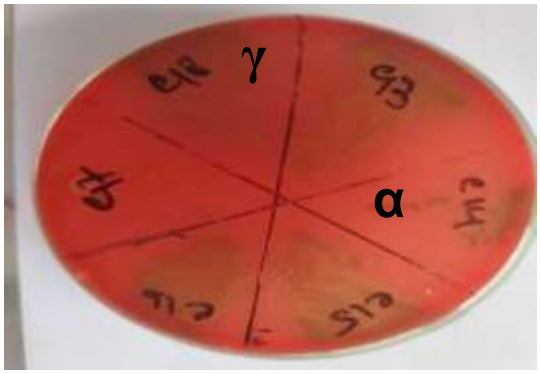

Figure 1. Hemolysis on blood agar (γ) isolate with gamma hemolysin, (α) isolate with alpha hemolysin

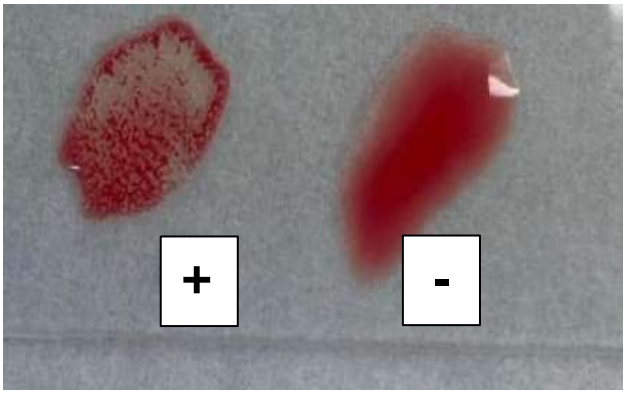

Figure 2. Hemagglutination test results (+) agglutination reaction of 1% of rabbit erythrocytes added to *E. coli* suspension, (-) no agglutination reaction occurred of 1% of rabbit erythrocytes added to *E. coli* suspension.

Figure 3. Results of bacterial suspension added with 0.8M Ammonium sulfate, (Right) Positive SAT result, (left) Negative salt agregation test result.

Table 1. Distribution of hemagglutination pattern of *E. coli* isolates from several animal's erythrocyte

	Erythrocyte group					
Total positive hemaglutination	Human	Cow	Cat	Dog	Rabit	Chiken
	0	21	0	0	27	0

Table 2. Distribution of virulence factors among *E. coli* isolates

Virulence factors		**Number**
Isolates having all three virulence factors		2
Isolates have Two Virulence factors	Haemolysin and haemagglutination	23
	Haemolysin and salt aggregation	0
	Haemaglutination and salt aggregation	0
Isolates have One Virulence factor	Haemolysin	1
	Haemagglutination	4
	Salt aggregation	0
Total		30

Based on the research results, 30 samples of *E. coli* isolated from *M. fascicularis* suffered diarrhea, overall had virulence factors. In 2 (6.67%) samples had three virulence factors, 23 (76.67%) samples had two virulence factors, and 5 (16.67%) other samples only had one virulence factor.

Escherichia coli produces three types of hemolysin, namely α, β and γ. Most *E. coli* produce alpha hemolysis compared to beta hemolysis and a small portion of gamma hemolysis (Schwidder et al. 2019; Walton and Smith,1969). Cytolytic protein toxins secreted by most isolated *hemolytic E. coli* are alpha-hemolysin and gamma-hemolysin. *Escherichia coli* alpha-hemolysin and gamma-hemolysin are exotoxins that are considered significant virulence factors in several clinical infections. Hemolysins are known to be toxic to a wide range of mammalian cells, and their production within host cells can significantly altered the cellular environment and local inflammation.

Detection of hemagglutinin in *E. coli* phenotypically usually used haemagglutination test using erythrocytes. Hemagglutinin is an important virulence factor as a marker of *E. coli* pathogenicity (Rajkumar *et al*., 2016). Detection of hemagglutinin in *E. coli* phenotypically usually used a haemagglutination test using erythrocytes. Bacteria agglutinated several different species of red blood cells such as humans, rabbits, guinea pigs, poultry, sheep, mice, etc. Haemagglutination of red blood cells by microbes is used as an epidemiological marker along with others. Several types of erythrocytes have been used in the haemagglutination test on *E. coli.* Rajkumar et al. (2016) used erythrocytes from various human blood groups, Khusnan and Purnomo, (2022) used erythrocytes from rabbits and humans Gupta et al. (1958) used erythrocytes from

humans, guinea pigs, and rabbits, and sheep, Mythreyi et al. (2011) used erythrocytes from cows and humans. In this research, the haemagglutination test used human, cow, dog, cat, rabbit, and chicken erythrocytes. Among the *E. coli* isolates examined, 21 (70%) exhibited hemagglutination of cow erythrocytes and 27 (90%) exhibited hemagglutination of rabbit erythrocytes, whereas none of the 30 isolates (100%) hemagglutinated human, dog, cat, or chicken erythrocytes. *E. coli* isolates that have hemagglutinin will be able to agglutinated erythrocytes, erythrocyte haemagglutination can occur when there is a bond between hemagglutinin on surfaced by *E. coli* and the hemagglutinin receptor on erythrocytes. Hemagglutination will not occur because of the differences in receptors on erythrocytes with the surface of bacteria. Based on research done by Evans *et al.,* (1980) and Hogan et al. (1990) reported the receptor differences on the surface of human, sheep, and chicken erythrocytes, cows with guinea pigs.

Cell surface hydrophobicity (CSH) is a biophysical measure of a cell's affinity for a hydrophobic versus hydrophilic environment. Cells with higher CSH prefered a hydrophobic environment while cells with lower CSH prefered an aqueous environment (Krasowska and Sigler, 2014). Salt aggregation test is a conventional method that is easy and often used to detect bacterial hydrophobicity (Ramesh et al. 2023). Based on the results of this study, 2 (6.67%) *E. coli* samples isolated from M. fascicularis suffered diarrhea cases were hydrophobic. The hydrophobicity of microbial surfaces plays an important role in bacterial adhesion to the host cell surface (Goswami et al. 2017), CSH is a cellular property that has broad effects on many aspects of microbial physiology. Bacterial adhesion to host cells and the ability to invaded cells are considered important steps in the infection process. Surface adhesion and subsequent biofilm formation are critical as they can lead to drug resistance (Mirani et al. 2018; Danchik and Casadevall, 2021). Rapid changes in environmental conditions forced adaptive modifications in microorganisms that enhanced their ability to survived (Krasowska and Sigler, 2014). The ability to formed biofilms may enhanced the survival of microorganisms, as cells growing in biofilms are highly resistant to components of the human immune system and many antimicrobial agents. Bacterial cell adhesins can be ranked based on their hydrophobicity, with pathogenic *E. coli* known to exhibited greater surface hydrophobicity than nonpathogenic *E. coli* (Nigudgi et al. 2023).

CONCLUSION

A total of 30 *E. coli* isolates expressed various virulence factors responsible for the diarrheal mechanism in *Macaca fascicularis.* Among these isolates, 24 isolates (80%) exhibited α-hemolysin, 2 isolates (6.67%) showed γ-hemolysin, and the remaining 4 isolates (13.33%) showed no hemolytic activity. In addition, 29 isolates (96.7%) exhibited hemagglutination toward bovine and/or rabbit erythrocytes, while 2 isolates (6.7%) showed positive hydrophobicity based on the salt aggregation test

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