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SEROTYPES, TOXINS AND ANTIBIOTIC RESISTANCE OF *Escherichia coli* (E.COLI) STRAINS ISOLATED FROM DIARRHEIC RABBITS IN PHU VANG, THUA THIEN HUE

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KEYWORDS

Antibiotic susceptibility

Diarrhea

E. coli

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ABSTRACT

This study was conducted to determine the prevalence of *E. coli* in rabbits, their biochemical and serological characteristics, common virulence genes, and antibiotic resistance. The diarrhea rabbit feces were collected from households and rabbit farms in Phu Vang - Thua Thien Hue with a total of 250 samples for testing. The results showed that rabbits age from 31 to 45 days old had the highest incidence of diarrhea disease caused by *E. coli* (92.0%) and the lowest infection rate was observed in rabbits over 60 days old with an infection rate of 30%. Further, the isolated *E. coli* strains tested biochemical characteristics showed 100% motile, positive for indole and methyl red, fermenting glucose and lactose. Simultaneously these strains were detected belong to 7 serotypes O103, O157, O158, O169, O44, O125, O153 and susceptible to cefuroxime (95.45%), akamicin (86.37%), streptomycin (81.82%), amoxicillin (81.82%), tetracycline (68.18%), colistin (68.18%), ampicillin (63.63%), gentamycin (59.10%) and levofloxacin (50.0%), whilst resistant to doxycycline (100%), sulfamethoxazole-bactrim (95.46%), and neomycin (86.37%). By using PCR assay for detection of virulence genes of the isolated *E. coli* strains, there were 7 strains carried virulence genes, of which 4/7 *E. coli* strains carried *eaeA* and *tsh* genes (57.14%), 2/7 strains carried *stx2* gene (28.57%); 1/7 *E. coli* strains carried *stx1* gene (14.28%) and the F4, F5 and F6 genes were not found in all serotypes in this study.

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1 Introduction

Rabbit farming in recent years has increased for various purposes including medical and educational laboratory animals. Rabbit meat is efficiently converted from vegetable protein into high-quality animal protein. However, the large-scale production of rabbit meat is severely affected by the high mortality of rabbits, which hinders mass production (Okerman, 1994). Gastrointestinal syndrome in young rabbits often has a clinical manifestation of diarrhea and leads to secondary infections, which is the cause of reduced resistance and increased mortality in rabbits, and serious economic impacts for the rabbit industry (Yang et al., 2017). *E. coli* is a very common bacteria in the gastrointestinal tract and it does not directly disease in rabbits, but stress or exposure to other pathogens can lead to its growth in the gut which results in death (Milon., 1996). Enteropathogenic *E. coli* (EPEC) is the main causative agent of acute intestinal disease in rabbits, characterized by intestinal lesions (Licois, 2004). Highly virulent *E. coli* strains causing diarrhea in rabbits were identified as belonging to 12 different O serotypes (Pisoni et al., 2004). Intestinal infections in rabbits caused by serotypes of O are known as diarrheal *E. coli* strains, so their pathogenicity is related to several virulence properties (Xia, 2010). In addition, the majority of *E. coli* isolates were resistant to different antibiotics (Wang, 2021). Currently, there is a lack of information on the incidence of diarrheagenic *E. coli* in rabbits in Vietnam. Therefore, in this study, we collected fecal samples from rabbit farms to identify the prevalence of *E. coli* in rabbits, biochemically, serologically, common virulence genes, and analyze their drug resistance characteristics to provide information to determine reasonable use of antibiotics.

2 Materials and Methods

2.1 Collection of samples

A total of 250 fecal samples were collected from 1 to 60 and over 60 days old New Zealand white rabbits with diarrhea from households and farms in Phu Vang–Thua Thien Hue, Vietnam. Collected samples were directly transferred to the Laboratory of immunology and vaccine, Institute of Biotechnology, Hue University for *E. coli* isolation and identification without any delay.

2.2 Isolation and biochemical identification of *E. coli*

The nutrient broth was used to enrich the collected samples and incubated for 20-24 hours at 37°C. Then inoculated separately from each sample onto Mac Conkey agar and further incubated for 24 hours at 37°C. Suspect colonies were selected for gram staining; morphological and biochemical characteristics of the isolated bacterial strains were determined according to Cruickshank et al. (1975).

2.3 Serological identification of isolated *E. coli* strains

The serotypes of isolated *E. coli* strains were determined by slide agglutination assay for detection of O antigen using *E. coli* antisera (Edwards & Ewing, 1972).

2.4 Antibiotic Sensitivity Test

The antibiotic susceptibility of isolated *E. coli* strains were determined by using the disk diffusion method as suggested by Bauer et al. (1966). Antibiotics used in this study including ampicillin (AM-10 µg), amoxicillin (AX-10 µg), colistin (Co-10 µg), gentamycin (GE- 10 µg), akamicin (AK-30 µg), streptomycin (SM-10 µg), tetracycline (TE-30 µg), doxycycline (DX-30 µg), sulfamethoxazole-bactrim (BT- 23.75 µg), cefuroxime (CU-30 µg), levofloxacin (LV-5 µg) and neomycine (NM-30 µg). The diameter of the zone of inhibition of *E. coli* bacteria by antibiotics on the diffusion disk was determined according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015).

2.5 Detection of common virulence genes of isolated *E. coli*

Genomic DNA of *E. coli* bacteria was extracted using the QIAamp DNA Mini Kit following the manufacturer's instructions. PCR conditions and primer sequences are shown in Table 1. The volume of the reaction was carried out in 25µl including (DNA template, primers of each, dNTP, PCR buffer, and Taq DNA polymerase). PCR products were separated by electrophoresis in 1.0% agarose gel with TAE buffer at 80V.

2.6 Statistical analysis

The data were analyzed using the Minitab statistical package version 14.0. The results were compared by X² test (Chi-square test) and analysis of variance (ANOVA), p-value < 0.05 was considered statistically significant.

3 Results

3.1 Prevalence of *E. coli* in rabbit samples

The prevalence of *E. Coli* isolated from rabbits with diarrhea in Thua Thien Hue is presented in Table 2. The results showed that an overall infection rate of 55.2%. Rabbits with the age group of 31-45 days old had the highest incidence of diarrhea caused by *E. coli* (92.0%), followed by rabbits of age between 16-30 days old, 46-60 days old, and 1-15 days old with an infection rate of 74.0 %, 48.0 %, and 32.0%, respectively. The lowest infection rate was observed in rabbits over 60 days old with an infection rate of 30%.

3.2 Results of isolation and biochemical identification of *E. coli*

The morphological characters of *E. coli* showed that all isolates were Gram-negative, rod-shaped, non-sporulated bacteria and arranged

Table 1 Conditions of PCR for virulence gene amplification

| Target Gene | Oligonucleotide sequences (5' - 3') | Size Fragment (bp) | Annealing Temperature (°C) | Reference |
|--|--|--------------------|----------------------------|---------------------------|
| Fimbrial (F4) | GAA TCT GTC CGA GAA TAT CA GGT ACA GGT CTT AAT GG | 505 | 53 | Boerlin et al., 2005 |
| Fimbrial (F5) | AAT ACT TGT TCA GGG AGA AA AAC TTT GTG GTT AAC TTC CT | 230 | 50 | |
| Fimbrial (F6) | GTA ACTCCACCGTTT GTATC AAGTTACTGCCAGTCTATGC | 409 | 53 | |
| Attaching and effacing (<i>eaeA</i>) | ATG CTT AGT GCT GGT TTA GG GCC TTC ATC ATT TCG CTT TC | 248 | 51 | Bisi-Johnson et al., 2011 |
| Temperature sensitive hemagglutinin (<i>tsh</i>) | AGT CCA GCG TGA TAG TGG AGT CCA GCG TGA TAG TGG | 620 | 54 | Delicato et al., 2003 |
| Shiga-Like Toxin (<i>stx1</i>) | ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG | 641 | 58 | Dipineto et al., 2006 |
| Shiga-Like Toxin (<i>stx2</i>) | CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTC | 779 | 58 | |

Table 2 Prevalence of *E. coli* isolated from diarrhea rabbits

| Days old | Number of examined samples | Number of positive samples | Percentage (%) |
|----------|----------------------------|----------------------------|----------------|
| 1-15 | 50 | 16 | 32.00 |
| 16-30 | 50 | 37 | 74.00 |
| 31-45 | 50 | 46 | 92.00 |
| 46-60 | 50 | 24 | 48.00 |
| >60 | 50 | 15 | 30.00 |
| Total | 250 | 138 | 55.20 |

singly or in pairs (Table 3). Bacterial colonies appeared as smooth, shiny, donut-shaped, and are surrounded by dark pink area, strong lactose fermenting colonies on MacConkey's agar. The typical biochemical reactions of isolated *E. coli* strains are lactose and glucose-fermenting colonies and positive tests for indole, methyl red, and motility; tests were negative for citrate administration, urea hydrolysis, voges-proskauer, and no H₂S generation (Table 3).

Table 3 Biochemical identification of isolated *E. coli*

| Biochemical test | Reaction |
|---------------------|----------|
| Glucose | + |
| Lactose | + |
| Motility | + |
| Indole | + |
| Methyl red | + |
| Vogus-proskauer | - |
| Citrate utilization | - |
| Urea utilization | - |
| Hydrogen sulphide | - |

3.3 Determination of serotypes of isolated strains of *E. coli*

The serotypes of isolated *E. coli* strains were determined by the slide agglutination test (Table 4). The results showed that from 45 isolated *E. coli* strains were classified into 7 serotypes including O103 (20.00%), O158 (20.00 %), O125 (15.55%), O153 (13.34%), O169 (13.34%), O44 (8.89%), O157 (6.66%) and Untypable (2.22%).

Table 4 Serotypes of isolated *E. coli* strains from diarrhea rabbits

| Serotypes | Number | Percentage (%) |
|-----------|--------|----------------|
| O103 | 9 | 20.00 |
| O158 | 9 | 20.00 |
| O125 | 7 | 15.55 |
| O153 | 6 | 13.34 |
| O169 | 6 | 13.34 |
| O44 | 4 | 8.89 |
| O157 | 3 | 6.66 |
| Untypable | 1 | 2.22 |
| Total | 45 | 100 |

Table 5 Susceptibility of isolated *E. coli* to 12 commonly used antibiotics

| Antibiotics | Number | Susceptible | | Intermediate | | Resistance | |
|---------------------------|--------|-------------|----------------|--------------|----------------|------------|----------------|
| | | No | Percentage (%) | No | Percentage (%) | No | Percentage (%) |
| Ampicillin | 22 | 14 | 63.63 | 6 | 27.28 | 2 | 9.09 |
| Amoxicillin | 22 | 18 | 81.82 | 3 | 13.63 | 1 | 4.55 |
| Colistin | 22 | 15 | 68.18 | 4 | 18.18 | 3 | 13.64 |
| Gentamycin | 22 | 13 | 59.10 | 0 | 0 | 9 | 40.9 |
| Akamycin | 22 | 19 | 86.37 | 2 | 9.09 | 1 | 4.54 |
| Streptomycin | 22 | 18 | 81.82 | 1 | 4.54 | 3 | 13.64 |
| Tetracycline | 22 | 15 | 68.18 | 2 | 9.09 | 5 | 22.73 |
| Doxycycline | 22 | 0 | 0 | 0 | 0 | 22 | 100 |
| Sulfamethoxazole -bactrim | 22 | 1 | 4.54 | 0 | 0 | 21 | 95.46 |
| Cefuroxime | 22 | 21 | 95.45 | 0 | 0 | 1 | 4.55 |
| Levofloxacin | 22 | 11 | 50.0 | 5 | 22.72 | 6 | 27.28 |
| Neomycine | 22 | 2 | 9.09 | 1 | 4.54 | 19 | 86.37 |

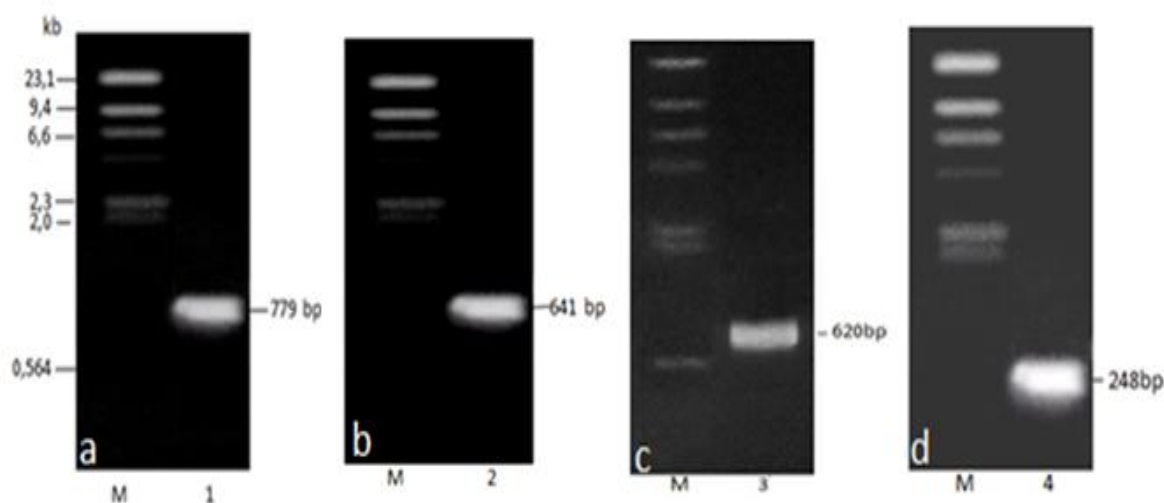


Figure 1 Detection of virulence genes of isolated *E. coli* strains by PCR on Agarose gel (a): *stx2* gene, lane 1 with 779 bp; (b): *Stx 1* gene, lane 2 with 641 bp; (c): *tsh* gene, lane 3 with 620bp; (d): *eaeA* gene, lane 4 with 248 bp; M; λ DNA/HindIII Markers.

3.4 Determination of antibiotic susceptibility of isolated *E. coli* strains

The antibiotic susceptibility of isolated *E. coli* strains was conducted with 12 antibiotics that are often used at rabbit farms in Vietnam. The results of the study showed that isolated *E. coli* have a higher sensitivity to cefuroxime (95.45%), akamycin (86.37%), streptomycin (81.82%), amoxicillin (81.82%), tetracycline (68.18%), colistin (68.18%), ampicillin (63.63%), gentamycin (59.10%) and levofloxacin (50.0%) while isolated *E. coli* were resistance to doxycycline (100%), sulfamethoxazole-bactrim (95.46%), neomycine (86.37%) (Table 5).

3.5 Detection of common virulence genes of isolation *E. coli* by PCR

A total of 44 isolated *E. coli* strains, only 7 different serotypes for each strain were tested by PCR to detect the presence of virulence genes including *eaeA*, *tsh*, *stx1*, *stx2*, F4, F5, and F6. The results showed that the tested *E. coli* strains had the presence of one of the virulence genes (*eaeA*, *tsh*, *stx1* and *stx2*) accounting for 100% (7/7). Whereas, the virulence genes F4, F5, and F6 were not found in all serotypes in this report (Table 6, Table 7, and Figure 1).

Table 6 The common virulence genes of isolated *E. coli* strains

| Virulence genes | <i>E. coli</i> isolates | Percentage (%) |
|-----------------|-------------------------|----------------|
| <i>eaeA</i> | 4/7 | 57.14 |
| <i>Tsh</i> | 4/7 | 57.14 |
| <i>stx1</i> | 1/7 | 14.28 |
| <i>stx2</i> | 2/7 | 28.57 |
| F4, 5, 6 | 0/7 | 0.00 |

Table 7 Virulence genes in serotypes of isolated *E. coli* strains

| Serotypes | Virulence genes | | | | |
|-----------|-----------------|------------|-------------|-------------|--------|
| | <i>eaeA</i> | <i>Tsh</i> | <i>stx1</i> | <i>stx2</i> | F4,5,6 |
| O103 | - | - | - | + | - |
| O157 | - | - | - | - | - |
| O158 | + | + | - | - | - |
| O169 | - | + | - | + | - |
| O44 | + | - | + | - | - |
| O125 | + | + | - | - | - |
| O153 | + | + | - | - | - |

4 Discussion and conclusions

E. coli is one of the common bacteria in the gut microbiota of rabbits, when the body is exposed to adverse conditions, they will become pathogens causing infections inside and outside of the digestive tract (Okerman, 1994). Rabbit farms are severely affected economically by the high mortality rate in rabbits caused by Enteropathogenic *E. coli* (Stakenborg et al., 2006). Diarrhea caused by *E. coli* leads to a high mortality rate in a rabbit farm with a rate of 75% (Hong et al., 2017). In this study, *E. coli* were isolated from the diarrheic rabbits with an overall incidence rate of 55.2% (Table 1). This number is similar to the findings of Alton et al. (2013), Entssar et al. (2000), and Sawsan (2012), those who have isolated *E. coli* from diarrheic rabbits with percentages of 53.7%, 61%, and 64%, respectively. In contrast, lower rates of *E. coli* from diarrheic rabbits were recorded by Sharada et al. (2010), Hasan et al. (2011), Literak et al. (2013), and Radwan et al. (2014) with 44.61%, 36.20%, 35.74%, and 41.5%, correspondently. In the current study, the highest incidence of diarrhea caused by *E. coli* was reported at the age of 31 to 45 days old, accounting for 92% (Table 2). The reason may be that at this stage, rabbits are often weaned, so their ability to be affected by adverse factors such as housing, food, and care conditions are high which lead to rabbits at a high disease rate (Dung, 2011, Bekheet, 1983; Shahin et al., 2011).

The classification of serotypes isolated from 44 *E. coli* strains are O103, O158, O125, O153, O169, O44, and O157 (Table 4). These serotypes are similar to O125 and O158 which were reported by

Aisha & Yousief (1999). Further, the serotypes O44 and O158 are associated with the diarrhea rabbits (Shahin et al., 2011) while serotype O125 is associated with the rabbit's enteritis (Alshimaa, 2007). Besides, the presence of different serotypes such as O111 and O114 were also reported by Scaletsky et al. (1984), serotypes O119, O55, and O128 by Morsy et al. (2002), and serotypes O109, O15, and O8 by Walaa & Lamyaa (2016). Some reports indicated that the common serotypes among the *E. coli* strain associated with rabbit diarrhea are O103, O49, O26, O128, O92, and O44 in which serotype O44 is present most frequently (Blanco et al., 1997; Marches et al., 2000; Morsy et al., 2002).

Regarding antibiotic sensitivity of isolated *E. coli* serotypes, out of 25 *E. coli* O serotypes identified, only 22 different serotypes were used for antibiotic susceptibility testing (Table 5). Results of antibiotic sensitivity showed variable sensitive to cefuroxime (95.5%), akamicin (86.37%), streptomycin (81.82%), amoxicillin (81.82%), tetracycline (68.18%), colistin (68.18%), ampicillin (63.63%), gentamycin (59.10%), and levofloxacin (50.0%). High resistance was reported against the doxycycline (100%), sulfamethoxazole (95.46%), and neomycine (86.37%). Similarly, *E. coli* strains isolated from diarrhea rabbits were resistant to sulfamethoxazole and susceptible to gentamycin (Moharam et al. 1993; Abd-El Rahman et al. 2005; Rhouma et al., 2020). In this study, all isolated *E. coli* showed high resistance to doxycycline, sulfamethoxazole, and neomycine. This is consistent with the fact that these antibiotics were widely used for the prevention and treatment of diseases in rabbits. Besides, they were very susceptible to cefuroxime, akamicin, streptomycin, amoxicillin, tetracycline, and different antibiotics. This suggests that these antibiotics are less frequently used in rabbit farms.

Out of 44 serotypes, only 7 isolates of *E. coli* were identified with different serotypes to detect the presence of common virulence genes by PCR (Table 6, 7). The results showed that the presence of the *eaeA* gene in serotypes O157, O158, O125, and O153 with 57.14% (4/7). Further, 57.14% (4/7) were positive to *tsh* gene present in serotypes O158, O169, O125, and O153; while 28.57% (2/7) were positive to *stx2* gene present in serotypes O103, O169; and 14.28% (1/7) were positive to *stx1* gene present in serotype O44. Camarda et al. (2003) reported that the *eaeA* gene accounted for 28.57% of the isolated *E. coli* strains tested by PCR, while Alexis & James (2003) reported that, 25% of the *eaeA* gene was present in a total of 28 samples tested, Rhouma et al. (2020) suggested that out of isolated 40 *E. coli* strains, 17 strains carry the *eaeA* gene. Similarly, Pohl et al. (1993) and Blanco et al. (2006) showed that the *eaeA* gene accounts for a high proportion in serotypes isolated from diarrhea rabbits. Hassan & Al-Azeem (2009) reported that 31% of the isolated strains of *E. coli* carried the *eaeA* gene and possessed an *stx1* gene. Mohamed et al. (2019) suggested that the serotypes of *E. coli* O148 and O44 isolated from

diarrheal rabbits carried the *eaeA*, *stx1* gene. Regarding, the *tsh* gene, Ashraf et al. (2014) detected in serogroups of *E. coli* O55, O125, and O146 possessing *tsh*, *eaeA* genes while virulence gene *stx2* was detected in serotypes O55 and O125. In this report, the presence of the serotypes carries virulence genes F4, F5 and F6 were not found in all serotypes in this study.

Conclusion

In this study, *E. coli* strains from diarrhea rabbits were isolated from households and farms in Thua Thien Hue, Vietnam. Isolated strains of *E. coli* were identified as belonging to seven serotypes including O103, O158, O125, O153, O169, O44, O157, and Untypable. The classified serotypes carry different virulence genes that cause diarrhea in rabbits. In addition, isolated strains of *E. coli* showed high resistance to some antibiotics commonly used in rabbit farms in Vietnam.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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