Exploring the phylogenetic group and clonal relationship Escherichia coli species obtained from hedgehogs in Kerman, Iran

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Received: October 2024 Accepted: January 2025

Abstract

ERIC-PCR technique is a simple, fast and cost-effective genotyping technology to detect different types of Escherichia coli species and genetic fingerprinting. The present study was designed and carried out with the aim of determining the phylogenetic group and clonal relationship of *Escherichia coli* isolates obtained from hedgehogs.

In the present study, 20 hedgehog feces samples were collected in Kerman city within 1 month and immediately sent to the veterinary faculty laboratory in less than 24 hours, and with the help of culture methods, one *Escherichia coli* isolate was obtained from each sample. After DNA extraction, PCR test was performed to trace the phylogenetic group of *Escherichia coli* isolates and to check the clonal relationship of the isolates by ERIC-PCR method.

In this study, among 100 isolates, 43 isolates belonged to phylogenetic group A, 52 isolates belonged to a phylogenetic group B1, 3 isolates belonged to group D, and 2 isolates were undetermined. Also, based on ERIC-PCR, these 100 isolates were grouped into 31 ERIC types with more than 98% similarity. Also, the strains in the porcupine population studied, which were related to a specific and limited geographical area, have a significant similarity to each other. *Escherichia coli* strains isolated from hedgehogs, have been shown to be mostly non-pathogenic in nature.

Keywords: *Escherichia coli*, Phylogeny, ERIC-PCR, Kerman, Iran.

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Introduction

Escherichia coli is one of the most diverse bacterial species. It normally lives in the intestines of healthy people and warm-blooded animals as a commensal or in water and sediments, and can also cause internal and external intestinal infections. Escherichia coli isolates are among the most common causes of diarrhea in humans and livestock, and based on virulence, phenotype, and pathogenicity factors, they are divided into various pathotypes, including enteropathogenic Escherichia coli (EPEC), enterotoxin-producing Escherichia coli (ETEC), and shigatoxin-producing Escherichia coli (STEC), enteric invasive Escherichia coli (EIEC), necrotoxic Escherichia coli (NTEC), enteric integrin Escherichia coli (EAggEC), and diffusive adherent Escherichia coli (DAEC) are divided (Tenaillon *et al*., 2010).

Many of the mentioned pathotypes have the ability to transmit and cause disease in humans and are therefore important. Some researchers believe that Escherichia coli pathogenic strains originated from opportunistic strains. This means that these bacteria Following They acquire pathogenicity by mutation or acquisition of chromosomal or plasmid virulence operons which results in the presence of different pathogenic clones in Escherichia coli populations. In terms of genetic structure, Escherichia coli is one of the bacteria in which recombination occurs to a small extent, and for this reason, it is widely used in investigating intraspecies polymorphism (Fegan *et al.,* 2014).

In evaluating the genetic evolution of Escherichia coli phylogenetic investigations are of particular importance, which are carried out by PCR, multilocus enzyme electrophoresis and ribotyping methods. Determining the phylogenetic groups is done with the help of different primers in PCR. Identification of the phylogeny group of Escherichia coli strains by PCR method is possible by determining the presence of chuA, yjaA, arpA, trpA genes and also a piece of DNA called TspE4.C2. Phylogenetic relationship of EcoR strains (Escherichia coli reference strain collection) shows that Escherichia coli strains are in seven main phylogenetic groups A, B1, B2, C, D, E, F and 5 clusters 1, 2, 3, 4 and 5 (Clermont *et al*., 2013) is placed.

Examining the genetic diversity of bacteria nowadays with the help of advanced and modern technologies, such as molecular diagnostic tools and genetic fingerprinting, can be a suitable choice in molecular epidemiological research. Polymerase chain reaction-based techniques are used for genetic fingerprinting due to their accurate, rapid, reproducible, sensitive, specific, and reliable diagnostics. Among several PCR-based tools, ERIC-PCR is a simple, rapid, and cost-effective genotyping technology for the detection of various species. In fact, ERICs are known as mobile DNA particles associated with small inverse transposable elements (MITEs) (Kotłowski *et al.,* 2013).

ERIC sequences belong to 126-base pair (bp) non-coding regions that are highly conserved and repeated several times throughout the bacterial genome. The location of ERIC sequences is different from one strain to another. Typing by ERIC-PCR was reported by Versalovich et

al. in 1991: This method is based on the oligonucleotides complementary to the ERIC sequences are attached. This may cause DNA to replicate between ERIC sequences, provided that the distance between ERIC sequences is less than 5 kb. Because there are multiple copies of the ERIC sequence, it is possible to generate multiple types of PCR products. Differences in the number and location of ERIC sequences between bacterial strains are expected to lead to observed differences in the number and size of PCR products between strains. This difference in the number and size of PCR products leads to differences in band patterns in electrophoresis of the products (Chulain *et al*., 2006).

Nowadays, the determination of phylogenetic groups of bacteria is useful for epidemiological studies (Carlos *et al*., 2010; Chomel *et al.,* 2007; Vidovic and Korber, 2016). In the study of Pajand *et al*. (2017) on the phylogenetic diversity of Escherichia coli clinical isolates obtained from patients, the most common phylogroup was reported to belong to phylogroup B2 and A. However, in two other studies that were conducted in France and China, in addition to B2, phylogroup A was one of the most common phylogroups in the samples obtained from urinary tract infection (Dubois *et al*. 2010; Luo *et al*., 2012). The results of past studies show that commensal isolates mostly belong to phylogroups A and B1, while the isolates that cause extraintestinal infections are mostly B2 and D (Adib *et al.,* 2014; Nowrouzian et al., 2006). In another study that was conducted on Escherichia coli isolates isolated from blood, phylogroups B2 and D were proposed as the most common phylogroups that show the highest level of resistance to antibiotics (Walk *et al*., 2007). In the study of Ghanbarpour *et al*. (2012) on the cases of human diarrhea, out of 96 investigated isolates, 52.1% belonged to group A, 1.2% belonged to group B1, 4.1% belonged to group B2 and 35.4% belonged to They were group D. Recent phylogenetic studies show that extraintestinal pathogenic Escherichia coli mostly belong to group B2 and to a lesser extent to group D, and the typing results confirm this finding (Skjøt-Rasmussen *et a*l., 2013). According to what was mentioned, the purpose of this research is to determine the phylogenetic group and clonal relationship of Escherichia coli isolates obtained from hedgehogs in order to identify the microbial population structure of the gastrointestinal tract of this animal in order to maintain the health and strengthen the immunity of hedgehogs against It is an infectious disease.

Materials and methods

Sampling, isolation, biochemical verification and storage the studied community consisted of all Escherichia coli strains that were isolated from hedgehog feces samples in Kerman city. In this study, 20 porcupine feces samples collected in Kerman city during 1 month were sent to the veterinary faculty laboratory in less than 24 hours. In the laboratory, the samples were cultured in McConkey's medium and after 24 hours of incubation at 37°C, the bacteria that had pink and smooth colonies in McConkey's medium were selected as Escherichia coli suspected isolates. From the culture of each sample, 5 single pink

colonies (lactose positive) were selected as Escherichia coli suspected isolates. Finally, in order to confirm, it was examined by biochemical tests including IMViC tests.

In order to store the confirmed isolates of Escherichia coli, each was cultured in 700 µL of Luria Bertani broth medium which was poured into a sterile microtube, and placed at 37°C for 24 hours. After the end of the incubation time, the microtubes were checked and if turbidity was observed, 350 microliters of 50% sterile glycerol was added to the contents of the microtubes and placed in the freezer at minus 20 degrees Celsius.

DNA Extraction

The boiling method was used for DNA extraction in this study. In this method, the stored bacteria were cultured in tryptic culture medium of soy agar and placed at 37°C for 16-18 hours. After the end of the incubation time, a single colony was used for DNA extraction. First, 400 microliters of sterile distilled water was transferred into sterile microtubes, and then a colony was slowly pipetted and mixed. The microtubes were placed in the heating block device at 98°C for 10 to 15 minutes.

Then, the microtubes were cooled for 5 minutes in the freezer at minus 20 degrees Celsius. Finally, the microtubes were centrifuged at 13,000 rpm for 1 minute. The microtubes were slowly removed from the device and 250 microliters of their supernatant was transferred into new sterile microtubes. At the end, the extracted DNAs were stored at minus 20 degrees Celsius for the next steps.

PCR test to trace the phylogenetic group of E. coli isolates

In this study, seven pairs of primers were used which were introduced in previous studies (Tenaillon *et al.*, 2010; Fegan *et al*., 2014; Clermont *et al.*, 2013). The PCR reaction was performed in a volume of 20 µL. To prepare the PCR mixture, for each sample, 10 microliters of master mix prepared by Pars Tos Company (Iran), 0.5 microliters of each primer and 3 microliters of the extracted DNA sample were poured into the microtube and filled with sterile distilled water. It was brought to 20 microliters, then the microtubes were placed inside a thermocycler (BIO RAD, United States) and the temperature program according to Table 1 was used to perform PCR test.

Table 1: Temperature program to perform PCR test.

Terminal elongation	lengthening	Primer binding	Vasirasht	Initial nature
72°C, 7 minutes	72°C, 30 seconds	55°C, 30 seconds	94°C, 30 seconds	94 °C, 5 min
1 time		Repeat 30 times		1 time

PCR test to check the clonal relationship of Escherichia coli isolates

All 100 Escherichia coli strains were selected for fingerprinting through ERIC-PCR (Enterobacterial Repetitive Intergenetic Consensus Sequencing-Polymerase Chain Reaction) to better understand the clonal relationship between

Escherichia coli strains. This method was performed using a pair of primers called ERIC1 and ERIC2, which was provided by Versalovich *et al*. (1991). At first, bacteria were cultured in LB liquid medium overnight at 37°C, DNA was extracted by commercial kits (Cinnagen, Iran), followed by ERIC-PCR test and finally electrophoresis (75 V) in 2% agarose gel for 3 hours. To evaluate the ERIC-PCR results, images of the bands were captured by the GelDoc 1000 imaging system (Vilbert Lormat, France). Band patterns of the images were calibrated and analyzed using 1D Pro software (Totalb, UK) and the similarity of ERIC variants was shown by drawing a phylogenetic tree using unweighted pairwise grouping with clustering arithmetic mean (UPGMA). In this method, ≥95% similarity cut-off was considered to identify isolates belonging to a clone.

Electrophoresis

All PCR products were electrophoresed on 1.3% agarose gel to identify the strains that were positive in terms of the desired genes and sequences. For this purpose, TBE buffer was first prepared, and after making agarose gel, the samples were electrophoresed in it. To prepare 1 liter of TBE buffer, 10.8 grams of Trisbase (molecular weight = 121.4) and 5.5 grams of boric acid (molecular weight = 61.83) were first dissolved in half a liter of distilled water and then dissolved in 75 0.0 g of EDTA (molecular weight = 372.2) was added to the solution and finally the volume of the solution was increased to one liter with distilled water. To prepare 100 cc of electrophoresis gel, 1.3 grams of agarose powder was dissolved in 100 cc of TBE buffer, and after heating and complete dissolution of the agarose powder in the buffer, 10-20 minutes were given to cool it, and then When the temperature of the mixture reached 55 degrees Celsius, 10 microliters of Fluorescent Fluorescent substance was added to it and spread inside a 20 x 20 cm dish containing the combs that create the well. After closing the gel, it was placed in an electrophoresis tank (Clever, UK). Next, 10 microliters of PCR products were poured into agarose gel wells. 5 microliters of DAN Ladder was poured into each well of the cassette. At the end, the samples were electrophoresed under a voltage of 120 volts for 45 minutes. In order to see the result of electrophoresis, the gel was examined with the help of GelDoc 1000 device and a photo was taken.

Results

Results of PCR test to trace the phylogenetic group of E. coli isolates

In this study, among 100 isolates, 43 isolates belonged to phylogenetic group A, 52 isolates belonged to phylogenetic group B1, 3 isolates belonged to phylogenetic group D, and 2 isolates belonged to no phylogenetic group (Fig. 1).

PCR test results to check the clonal relationship of Escherichia coli isolates

In this study, all 100 Escherichia coli isolates obtained from hedgehogs were all genetically fingerprinted by ERIC-PCR method and the patterns obtained after electrophoresis were calibrated and analyzed with the help of 1D Pro software (Totalb, UK). and the similarity of ERIC types was shown by drawing a phylogenetic tree with the UPGMA algorithm (Fig. 2).

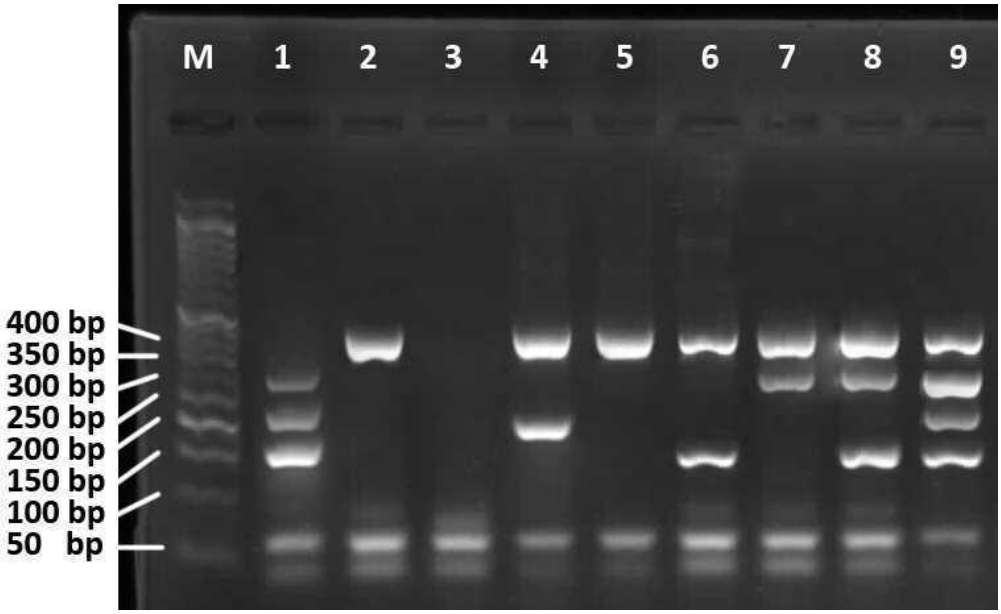

Figure 1: **Electrophoresis gel image related to PCR of isolates for their phylogenetic grouping; M: Marker, 1: positive control (EcoR62) for (288bp) chuA, (211bp) yjaA and (152bp) TspE4.C2 genes, 2: positive control (EcoR20) for (400bp) arpA gene, 3: negative control, 4: Phylogenetic group A or C, 5: Phylogenetic group A, 6: Phylogenetic group B1, 7 and 8: Phylogenetic group D or E, 9: Undetermined phylogenetic group.**

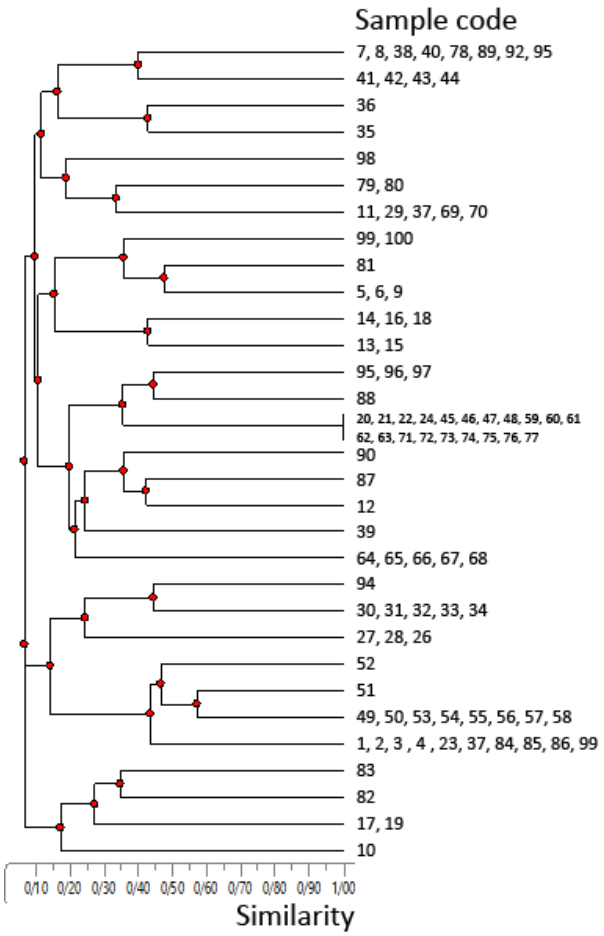

Figure 2: **Phylogenetic tree based on UPGMA algorithm based on ERIC-PCR patterns in this study**.

In this method, with ≥95% similarity cutoff, 31 clones were identified, which indicates the placement of different isolates from different hedgehogs in the same clone, and this in turn can indicate the rotation of similar strains. Among the different hedgehogs in the study area.

In this picture, 31 terminal branches represent 31 clones. The code of the isolates that are placed in each clone is written at the end of each branch. For example: In the first clone, isolates 7 and 8 belong to hedgehog #2, isolates 38 and 40 belong to hedgehog #8, isolate 78 belongs to hedgehog #16, isolate 89 belongs to hedgehog #18, isolates 92 and 95 belonging to hedgehog number 19 are all placed in the first clone.

Discussion

Escherichia coli is usually found in the intestinal microflora of warm-blooded animals. This species can be assigned to one of the four main phylogenetic groups, A, B1, B2 and D, and to seven subgroups A0, A1, B1, B22, according to the presence of a combination of three genetic markers chuA, yjaA and DNA fragment TspE4.C2. Divided B23, D1 and D2. The results of Carlos et al.'s study (Carlos *et al*., 2010) showed that group B1 strains were present in all hosts examined, but were more common in cattle, goat and sheep samples. Subgroup B23 was only found in human samples. Murphy *et al*. (2021) stated that although the genomes of Escherichia coli in wild animals and human gut are well separated, they contain pathogenic and antibiotic-resistant genes, and wild animal Escherichia coli can serve as reservoirs for pathogenicity. Escherichia coli act in human and livestock infections. Nowadays, determining the phylogenetic groups of bacteria is useful for epidemiological studies, in fact, by using molecular phylogenetic classification and determining the resistance and sensitivity pattern of Escherichia coli, the spread of many resistant infections can be prevented with appropriate antibiotic treatment. The economy and health of different communities helped a lot (Smati *et al*., 2015).

The results of past studies show that commensal isolates mostly belong to phylogroups A and B1, while the isolates that cause extraintestinal infections are mostly of B2 and D types. Phylogroups B2 and D are mostly isolated from the environment, and Escherichia coli isolates that are isolated from extraintestinal diseases belong to these two groups (Adib *et al.*, 2014). Despite the fact that the two phylogenetic groups B2 and D have more pathogenic properties and several reports have shown that Escherichia coli commensal isolates belong to groups A and D, but there are reports indicating the dominance of phylogroup A among urinary isolates. (Nowrouzian *et al*., 2006). In another study that was conducted on Escherichia coli isolates isolated from blood, phylogroups B2 and D were proposed as the most common phylogroups that show the highest level of resistance to antibiotics (Walk *et al*., 2007). In a study for phylogenetic typing of urine samples, it was shown that 65% of the isolates are in group B2, 19% in group D and 16% in group A, and none of them were in group B1 (Etebarzadeh *et al*., 2012). In another study on broiler poultry suffering from general bacillosis in Tabriz city, out of 70

isolates, the highest amount of isolated phylogenetic group was reported to belong to group A (Rodriguez-Siek *et al.,* 2005). Jia et al. reported in 2011 that poultry meat is one of the main agents for the transmission of E. coli from poultry to humans (Xia *et al.,* 2011). Recent phylogenetic studies show that extraintestinal pathogenic Escherichia coli mostly belong to group B2 and to a lesser extent belong to group D, and the typing results confirm this finding (Skjøt-Rasmussen *et al.,* 2013). One of the reasons for these differences could be the differences in the geographical distribution of microorganisms.

The results of the study by Sohrabi and Zaighami (Sohrabi and Zeighami, 2016) on the determination of phylogenetic groups in uropathogenic and commensal Escherichia coli isolates using the PCR method showed that the frequency of phylogenetic groups B2 and D in the isolates under study was high. Therefore, it can be stated that the mentioned isolates had a high pathogenicity; Because the results of different studies indicate that these two phylogenetic groups carry more virulence factors in comparison with phylogenetic groups A and B1. In the mentioned study, the evaluation of the phylogenetic groups of uropathogenic Escherichia coli strains showed that the highest frequency related to B2, D and A groups was 67.1%, 21.2% and 11.7% respectively. The comparison of these results with the studies of Prafidzadeh et al. in 2012 in Tehran (Etebarzadeh *et al*., 2012), Sabate *et al*. (2006) in Spain, Kawamura *et al*. (2010) in Japan in terms of the frequency of B2 groups, D and A matched. However, it was not similar to the study of Moreno et al. in 2009, where D and A groups were known to be dominant respectively (Moreno *et al*., 2009). Generally, different percentages of phylogenic groups have been obtained in different studies conducted in different parts of Iran and the world.

In the study of Ghanbarpour et al. (2010) on Escherichia coli strains isolated from poultry salpingitis, the highest frequency of phylogenic groups A and D (75%) was reported. In Hossein *et al*.'s study (2013), 153 poultry pathogenic Escherichia coli strains and 30 poultry faecal Escherichia coli strains were phylogenically investigated. Jeong *et al*. (2012) investigated the phylogenic groups in Escherichia coli isolated from chickens with colibacillosis and reported that 39.5% of the isolates with the highest frequency belonged to group A, which is the most important group of extraintestinal pathogens in humans. and for this reason, poultry were recognized as an important factor in the transmission of these strains to humans (Jeong *et al*., 2012). In the phylogenic study of Carvalho et al. in 2016 on Escherichia coli strains isolated from dogs and their owners, it was shown that group A was the most abundant in both groups and the strains isolated from both groups showed great similarity. is (Carvalho *et al*., 2016). In the study of Derakhshande *et al*. (2013) on human urinary pathogenic Escherichia coli strains, 67.64% belonged to group A and 17.64% and 14.7% of isolates belonged to B2 and D groups, respectively. in the study of Leo et al. in 2014 on Escherichia coli samples isolated from mastitis samples in cattle, group A was the most frequent (Liu *et al*.,

2014). Darehabi *et al*. (2013) also investigated Escherichia coli strains isolated from frozen foods of animal origin and strains isolated from children's diarrhea. In this study, the most phylogenetic group found in both groups was phylogenetic group A, which can indicate the transmission of this strain from food consumed to children.

Conclusion

In this study, among 100 isolates, 43 isolates belonged to phylogenetic group A, 52 isolates belonged to a phylogenetic group B1, 3 isolates belonged to group D, and 2 isolates were undetermined. Also, based on ERIC-PCR, these 100 isolates were grouped in 31 ERIC types with a similarity of more than 98%. From this study, it can be concluded that Escherichia coli strains isolated from hedgehogs are mostly non-pathogenic in nature and these strains have a significant similarity in the studied hedgehog population, which is related to a specific and limited geographical area to each other.

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