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A N I N T E R N A T I O N A L J O U R N A L

Evaluation of Genetic Analysis of *ESCHERICHIA COLI* Isolated from Two Different Environmental Sources: Sewage Water Verses Soiled Bedding Materials of Laboratory Rodents

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ABSTRACT

The present investigation details an assessment of genetic relationship of E. coli isolates collected from two different environmental sources viz. sewage water and soiled bedding materials of laboratory rodents. Five sewage water samples were collected from the industrial area of Lucknow city and 5 samples of soiled bedding materials of laboratory animals were collected from Animal facility at CSIR-IITR, Lucknow. For this study Random amplified polymorphic DNA markers (RAPD) was chosen as the molecular fingerprinting method. In this study, 10 RAPD primers were used to evaluate the genetic similarity of E. coli. isolates. The RAPD-PCR fingerprints were analyzed and data were scored as 1, 0 matrix. The generated data were fed on Popgene software for calculating genetic diversity and creating dendrogram. The genetic similarity of 85% was recorded from soiled bedding materials and only 71% in sewage water samples in E.coli samples. The dendrogram based generation of clustering of E. coli isolates show two major clusters. Within major cluster sub-cluster were also observed which indicating diversity within isolates of E. coli. The RAPD-PCR based fingerprinting provided a rapid means of discriminating E. coli isolates and considered a relevant tool for molecular typing.

Key words: *Escherichia coli*, Genetic similarity, RAPD-PCR and Rodents

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INTRODUCTION

The *Escherichia coli have* been intensively studied under various aspects in general bacteriology. *E.coli* is a common inhabitant of the gastrointestinal tract of most animals, including birds, but not all *E.coli* isolates are capable of causing diseases [1]. Analysis of *E.coli* strains was isolated from chicken by Random Amplified DNA marker [2]. The pathogenic *Escherichia coli*, gram negative bacteria, have been widely studied under different biomedical research like vehicles for the cloning of genes, testing of efficacy of antimicrobial agents, indicator organisms, etc. DNA fingerprinting of the species of *Escherichia* and *Shigella* revealed that *E. coli* is very closely related to *Shigella* [3]. The bacteriological examination of water has a special significance in pollution studies, as it is a direct measurement of deleterious effect of pollution on human health [4]. Differentiation of *Escherichia coli* strains using randomly amplified polymorphic DNA analysis and protein biochemical markers were studies [5]. Identification and differentiation of E.coli strains were isolated from clinical samples by RAPD marker [6]. The other genotyping studies carried out on clinical, environmental and veterinary isolates, using *E. coli* previously identified via biochemical and/or culture based approaches [7]. Isolation and identification of *Escherichia coli* O157:H7 using different detection methods and molecular determination by multiplex PCR and RAPD [8]. Using of multiplex PCR for Detection of *Escherichia coli* O157:H7 and analysis of RAPD, PFGE, plasmid profiling and antimicrobial property [9]. Isolation of Escherichia *coli* O157 strain and their molecular characterization isolated from cattle, pigs and chickens at slaughter [10]. In our previous study the evaluation of genetic analysis of rodents performed by RAPD techniques. [11]. The RAPD analysis uses single primers to amplify the genomic DNA as compare to restriction fragment length polymorphism (RFLP). Each primer can generate a specific DNA profile per sample [2]. Genetic characterization of *Escherichia coli* isolates from humans and animals sample by RAPD marker [12]. The RAPD analysis, ribotyping and serotyping of *E.coli* an epidemiological study was carried by Vogel et al., (2000) [13]. Some studies were also done in isolation of *E.coli* from house mice (wild mice) [7]. Some studies in isolated and screening of *E.coli* from different location in Gomati river Lucknow city have also been conducted [4]. Few studies have been conducted on genetic diversity of pathogenic*Escherichia coli* from different species of laboratory rodents by RAPD marker [14]. Ultimately there are few studies done on to the pathogenicity of E.coli strains from industrial site of sewage water sample and compared with laboratory animals (rodents) soil bedding material because the sewage water directly falls into Gomati river and contaminates the water of Gomati river which causes different diseases to among humans as well as animals. The Gomati river is an important river which passes from Lucknow city and is also a main source of drinking water supply. Gomti river receives huge quantities of untreated sewage agricultural runoff which brings lot of pesticides, pathogen microbes especially of *E.coli*. *Shigella* and *salmonella* species; industrial wastes significantly alter the physicochemical characteristics of its water. Very few studies has been conducted and there is scant literature on phylogenetic relationship of *E. coli* strains in bedding material of different laboratory rodents and sewage water from Gomati river [7]. While monitoring of rodents from animal house facility and Gomati River, the pathogenic *E.coli* strains play an important role in colony management of Gomati river and laboratory animal facility.

The aim of this study was to evaluate the genetic diversity of E.coli from two different Environmental Sources, Therefore 10 RAPD primers were used for the genetic diversity and molecular characterization of E.coli strains from two different places in Lucknow city India.

The RAPD technique provides a simple, fast and a comparatively low-cost marker system which has gained wide acceptance, world-wide [15]. In the present investigation of this study was to evaluate the genetic analysis of Escherichia coli isolated from two different environmental Sources: Sewage Water Verses Soiled Bedding Materials of Laboratory Rodents based on RAPD markers.

MATERIALS AND METHOD

Bacterial culture and growth condition

The present study was carried out at Environmental Microbiology Section CSIR-Indian Institute of Toxicology Research Lucknow India. The samples are collected from possible distinct environmental conditions. Five water samples and five bedding samples were taken initially for analysis of *E.coli* strains: such as from sewage area near Gomati River and animal facility room in IITR Lucknow.

In media preparation the nutrient broth was prepared by dissolving 13 gram of dehydrated nutrient broth (Himedia, India) into 1000 ml of distilled water and was sterilized by autoclaving at 121^0C under 15l b pressure per square inch for 15 minutes. Then the broth was dispensed into tubes (10 ml/tube) and stored at 4^0C in the refrigerator until used. The protocol for growing the E.coli bacteria was followed with Red hot sterilized platinum loop bein used to streak the nutrient broth culture on EMB agar or MacConkey agar for isolating colony. The media containing streaked culture was kept at 37^0C for overnight in incubator. The colony of pink/red and greenish black with metallic sheen appears on MacConkey and EMB agar plates respectively.

Biochemical Identification

There are different biochemical test to use to identify the *E.coli* bacterial strains like-

Catalase Test: Using a glass capillary tube, small amount of culture from the plate was used. Care was taken to ensure that no blood strains were present as the presence of catalase in the medium itself may give a false positive result. To the observed the immediate formation of oxygen bubbles in the tube indicating the activity of catalase. Each culture was observed for the appearance of or absence of gas bubbles.

Iodole Test: Bacterium to be tested is inoculated in peptone water, which contains amino acid tryptophan and incubated overnight at 37^0C. After incubation few drops of Kovac's reagent were added, Kovac's reagent consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol and conc. HCL. Ehrlich's reagent is more sensitive in detecting indole production in anaerobes and non-fermenters. The tube color was examined in the reagent "layer".

Methyl Red (MR) Test: The bacterium to be tested in inoculated into glucose phosphate broth, which contains glucose and phosphate buffer and incubated at 37^0C for 48 hours. Over the 48 hours the mixed acid producing organism should produce sufficient acid to overcome the phosphate buffer and remaining acid. The pH of the medium is tested by the addition of 5 drops of MR reagent. The change in color of methyl red or MR test was observed.

Voge'sProskauer (VP) Test: Bacterium to be tested is inoculated into glucose phosphate broth and incubated for at least 48 hours. 0.6 ml of alpha-naphthol is

added to the test broth and shaken. 0.2 ml of 40% KOH is added to the broth and shaken. The tube is allowed to stand for 15 minutes. The negative tubes must be held for one hour, since maximum color development occurs within one hour after addition of reagents. Observe the tubes for change in color for the VP test.

Citrate Utilization Test: Preparation of Simmon's citrate agar slants. Inoculate Simmon's citrate agar slants, by means of stab-and-streak inoculation. The one tube is kept as an un inoculated comparative control. Incubate all the three slants at 37^0C for 48 hours. Observe the slant cultures for the growth and coloration of the medium.

Gram's stain: In gram's staining under microscope the organism revealed gram-negative, pink color, small rod shaped, single or paired. The representative figure of Gram's staining is as follows.

Identification and DNA extraction of E.coli strains

After biochemically and staining processes, 10 samples were identified as containing *E.coli* colonies than confirmed pathogenic *E. coli* species were isolated from sewage water sample and bedding material of laboratory animal species. DNA was extracted from *E. coli* after inoculation in Luria broth followed by incubation at 37°C in tail digestion buffer and 20% SDS buffer with 5 µl proteinase K followed by phenol: chloroform: isoamyl extraction and ethanol precipitation with 3 M Sodium acetate according to Sambrook and Russell (1989)[16] protocol. Ten samples (five from sewage water sample and five from bedding material) from each sources were screened for *E.coli* isolate and subsequently DNA was extracted and stored at -20 ^{o}C for future use.The quantity of genomic DNA was estimated by spectrophotomer and DNA samples loaded on 0.8 % agarose gel.

RAPD-PCR

A total of 15decamer primers from the set OPA, OPG and OPO (Operon Biotechnologies GmbH, Germany) having GC content 60–70 % were initially screened RAPD PCR. The oligonucleotide primers information along with amplified band size range and GC content is provided in Table 1.The RAPD-PCR reactions were performed in a mixture of 25 µl volume containing 50 ng of genomic DNA , 12.5 µl hot start master mix (Biotool)), 1.0µL (25 pmole) primer and volume make up nuclease free water. DNA was amplified by a thermal circler (mycycler, BioRad^{TM}, USA) using the following cycling conditions: first denaturation of 5 min at 94°C followed by 45 cycles of 1 min at 94°C, 1 min at 35°C and 1 min at 72°C and final extension at 72°C for 5 min. One negative control was used in each primer reaction. The PCR reactions were carried out as described by Maity and Guru (2006) [14]. The PCR amplified products were resolved on 1.75 % agarose gel having ethidium bromide in 1X TBE buffer [45 mMTris–borate, 1.0 mM EDTA (pH 8.0)] at 75 V for 4 -5 h . A low range ladder was used for molecular weight determination. The amplified band pattern were visualized and photographed in a gel documentation system using Gene snap software (Syngene), UK.

Table 1. Primer name, Sequence, GC content, Tm value ^{O}C, Size of amplicons and Number of bands scored.

No.	Primers	Sequence (5' 3')	G+C (%) content	Tm value°C	Size of amplicons (bp)	No. of bands scored
1.	OPA 03	AGTCAGCCAC	60%	32.0	250-3000	11
2.	OPA 12	TCGGCGATAG	70%	34.0	300-2800	14
3.	OPA 13	CAGCACCCAC	70%	34.0	250-3250	13
4.	OPA 20	GTTGCGATCC	60%	32.0	200-2500	8
5.	OPG 01	CTACGGAGGA	60%	32.0	250-3000	13
6.	OPG 03	CTAGTGCTAC	50%	30.0	250-3000	8
7.	OPG 06	GTGCCTAACC	60%	32.0	200-2500	9
8.	OPG 07	GAACCTGCGG	70%	34.0	220-2700	11
9.	OPG 08	TCACGTCCAC	60%	32.0	350-2900	7
10.	OPG 11	TGCCCGTCGT	70%	34.0	225-2100	11
11.	OPG 12	CAGCTCACGA	60%	32.0	300-2400	9
12.	OPG 13	CTCTCCGCCA	70%	34.0	350-2850	8
13.	OPG 16	AGCGTCCTCC	70%	34.0	335-2700	9
14.	OPO 08	CCTCCAGTGT	60%	32.0	250-2850	8
15.	OPO 12	CAGTGCTGTG	60%	32.0	270-4800	15

RAPD data analysis

The analysis of allele size amplified by different RAPD markers was done in Genesnap and Gene tool softwares (Syngene, UK) along with the standard size ladders (100 and 500 bp).After matching the bands pattern in different gels than bands were scored as '1' and '0' for their presence and absence respectively. For molecular genetic investigation the binary coded characters (1, 0) were used. From generated binary data, the level of polymorphism was analysed by dividing the polymorphic bands by the total number of scored bands. Similarly, % of polymorphic loci was calculated for each genus using popgene version 1.31 software [17].

RESULTS

On the basis of culture examination and biochemical parameter of isolated bacteria from Gomati sewage water and soiled bedding material of laboratory animals are given bellow.

Biochemical test

The samples were collected and promptly inoculated on McConkey agar plate. After overnight incubation, bright pink colonies were observed in 10 Gomati River and soil bedding material samples. The presumptive colony on McConkey agar for each bedding material was sub cultured successively onto Eosin Methylene Blue (EMB) agar for presumptive identification of *E. coli*. The greenish-black colonies with metallic sheen on EMB agar were observed. In gram's staining under microscope the organism revealed gram-negative, pink color, small rod shaped, single or paired. Presumptively selected 2 to 3 mm colonies were repeatedly streaked on the respective selected media (EMB agar) to check and confirm their purity. For identification, a series of biochemical tests especially selective for *E. coli* were performed with the culture positive and gram-negative rod shaped cells. All the isolates are fermented the two sugars producing acid and gas. Acid production is indicated by the color change from reddish to yellow and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tubes.

PCR product analysis

In this study, 20 RAPD primers for amplification of bacterial genomic DNA were selected from two different sources. Out of 20 Only 12 primers formed the best, suitable, clear, and reproducible banding patterns. The amplified band patterns were found to be clear and reproducible when reaction were repeated using the same reaction condition. The total 165 types of bands were amplified by 12 RAPD primers i.e. scorable and easily counted. All 20 RAPD primers yielded reproducible DNA profiles. Control assays in which distilled water was used in place of template yielded no amplified products. The polymorphism was counted in different *E.coli* strains from different sources, some bands similar and some bands dissimilar reported. The primer OPG-02, OPG-07 and OPG -14 produced good results for investigation of polymorphism in all strains. The primer OPG-02 and OPG-07 approximately 250 bp and 600 bp allele size were similar in all strains of *E.coli*. (Fig. 1).The primer OPG-12 and OPG-07 produced good polymorphism in all strains of E.coli bacteria (Fig. 2&4). The numbers of scored bands, band size ranges were represented in Table 2. The molecular weight of amplified bands ranged from 200 bp to 3.4kbp. The maximum size-range of amplified products (230– 2,400 bp) for a single primer was found with OPG- 02, whereas the minimum (150–1,900 bp) was obtained with OPG-07. The maximum number of bands (11) was produced with primer OPG-02 (Fig. 3) and the minimum (1) with primer OPG-06. The RAPD DNA finger printing with 20 primers generated a total of 2,120 bands in all samples.

Table 2. Biochemical test of all sample of *E .coli*.

Catalase test	Iodole test	MR test	VP test	Citrate utilization test	Sugar test				
					Dextrose	Sucrose	lactose	Maltose	Mannitol
+	+	+	-	-	+	+	+	+	+

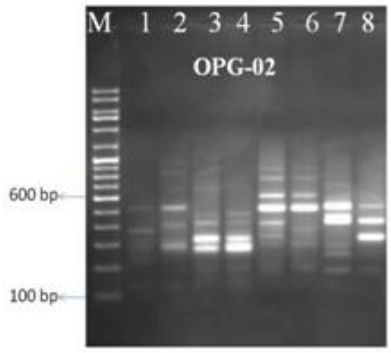

Fig.1. RAPD-PCR gel patterns of primer OPG-02 showing *E. coli* samples. Lane M is 100 bp molecular weight marker ladder, lane 1–4 E.coli from Gomati river samples and lane 5– 8 E.coli from soil bedding material samples.

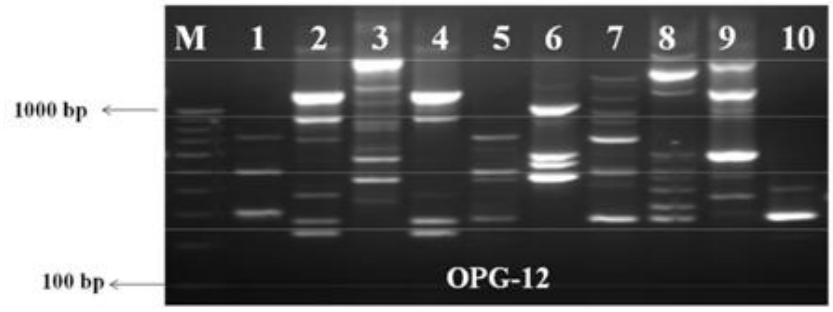

Fig. 2. RAPD-PCR gel patterns of primer OPG-12 showing E.coli samples. Lane M is 50 bp molecular weight marker ladder, lane 1–5 E.coli from Gomati river samples and lane 6– 10 E.coli from soil bedding material samples.

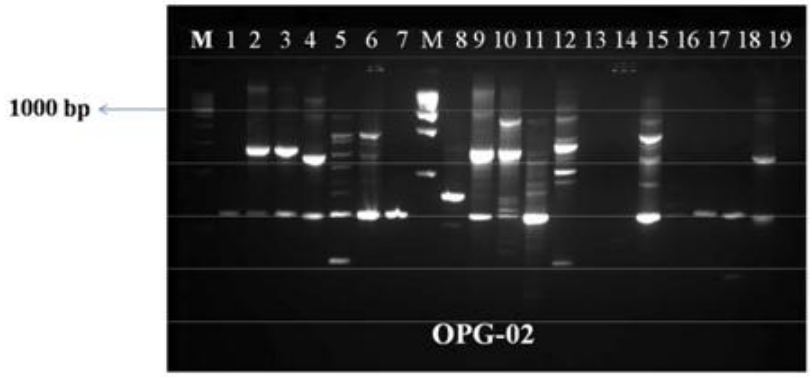

Fig.3. RAPD-PCR gel patterns of primer OPG-02 showing E.coli samples. Lane M is 100 bp and 1 kbp molecular weight marker ladder, lane 1–10 E.coli from Gomati river samples and lane 11–119 E.coli from soil bedding material samples.

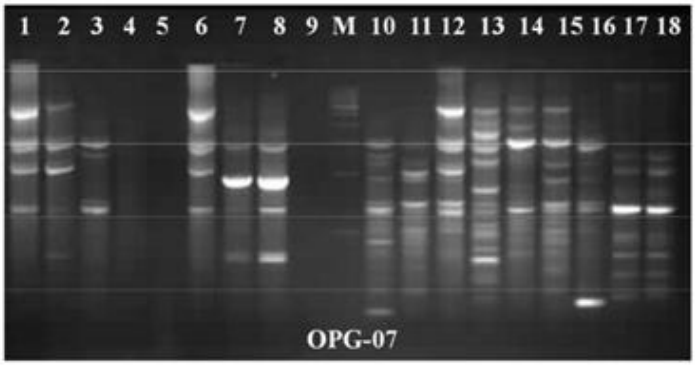

Fig. 4 RAPD-PCR gel patterns of primer OPG-07 showing E.coli samples. Lane M is 100 bp molecular weight marker ladder, lane 1–9 E.coli from Gomati river samples and lane 10 –18 E.coli from soil bedding material samples.

Phylogenetic tree analysis

In the dendrogram analysis of all E.coli Strains are grouped into two major clusters A and B. The cluster A contains E.coli strain isolated from Gomati River and cluster B contain soil bedding material of laboratory animals. The cluster A further separated in five different sub clusters isolated from Gomati River likewise cluster B separated also separated in five different sub culture isolated from soil bedding material from laboratory animals (**Fig. 5**). The genetic similarity of 85% was seen in *E. coli* samples isolated from soiled bedding materials and only 71 % were observed in sewage water samples .The Nei's genetic similarities and genetic differences were among all E.coli Strains were given in Table 3.

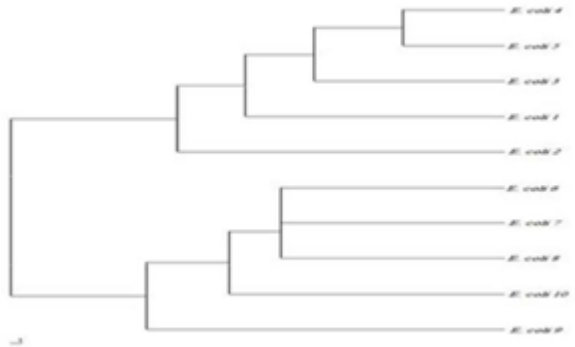

Fig. 5. Dendrogram analysis of E.coli samples constructed by Pop gene software.

Table 3. Genetic similarity (above diagonal) genetic distance (below diagonal) Sample 1-5 *E.coli* isolated from sewage water and 6-10 soil bedding material of laboratory rodents.

Sample	1	2	3	4	5	6	7	8	9	10
1	***	0.60	0.64	0.60	0.65	0.44	0.36	0.60	0.36	0.52
2	0.51	***	0.56	0.60	0.44	0.28	0.44	0.52	0.50	0.44
3	0.44	0.57	***	0.72	0.72	0.48	0.48	0.48	0.56	0.64
4	0.51	0.65	0.32	***	0.84	0.36	0.36	0.44	0.36	0.52
5	0.38	0.51	0.32	0.17	***	0.28	0.28	0.28	0.44	0.52
6	0.82	0.82	0.73	0.92	0.98	***	0.36	0.68	0.68	0.52
7	0.92	0.98	0.73	0.82	0.93	0.38	***	0.68	0.52	0.54
8	0.51	0.82	0.74	0.82	0.82	0.38	0.38	***	0.52	0.68
9	0.92	0.65	0.57	0.94	0.82	0.51	0.65	0.65	***	0.52
10	0.65	0.82	0.44	0.65	0.65	0.65	0.38	0.38	0.65	***

DISCUSSION

In our study, the genetic diversity of E.coli strains was investigated by RAPD PCR techniques. Evaluation of genetic diversity among different E.coli strains at genomic level was analysed by RAPD PCR technique [18-19]. Several arbitrary primer based RAPD-PCR technique has been used for delineating the bacteria according to their genetic relatedness [20-22]. In this study reveals with DNA marker RAPD-PCR based genetic analysis of different *E.coli* strains was compare with difference place of other *E.coli* strains [14]. In previous year there is no evidential data were found to be differentiate to the E.coli strain in different region using RAPD technique. In this study we monitored the Gomati River in Lucknow city and bedding material Laboratory animal. However, we believe that the genetic analysis of E.coli based on PCR method evaluate in this paper, may offer a cost-effective and time saving of E. coli isolates from different type of sample. The amplified fragment size of E.coli strain was investigated to the band size average between 200 bp to 3.4 kbp. The E.coli is the most important species in the genus *Escherichia* and recognized as an important potential pathogenic in humans as well as animals [23]. The other types of study were carried out in some animal species such as Identification and differentiation of pathogenic *E. coli* strains in animals using RAPD-PCR, multiplex-PCR [24-26]. The Result showed that the significant effect of E.coli in different places such information indicate that the Gomati river isolates will be more diversity when compare to soil bedding material isolates. The primerOPA-12 (Fig. 3) having GC content 70 % produces highest number of bands in all the E.coli strains. The results agreements with other types of study of *E.coli* in laboratory rodents were investigated [14]. The most challenging aspect of a protocol for E.coli diversity by RAPD study was the selection of suitable primers. We found that primers OPO 12, OPA 12, OPA13, OPG 01, OPG 07 and OPG 11 generated totally different banding pattern for each *E.coli* and could differentiate all the strains from one another. A phylogenetic tree was constructed by popgene software in all *E.coli* samples based on RAPD marker. These dendrogram generated from different primers were separated from one another, suggestive of genetically different patterns in different places. The similar type of study was also reported by [14]. The genetic similarities and genetic distance was found to be more informative to know the diversity of E.coli isolated from two different places. From this of study, it is concluded that RAPD markers were useful in detecting, genetic similarities, genetic diversities and genetic distances in different pathogenic of *E.coli* strain in different places.

These results also help to the understanding of the working on *E. coli* species in laboratory rodents in biomedical research. Thus, we suggest that application of this type of study in molecular characterization of *E.coli* infections to understand the

pathogenic and diversity of the bacteria isolated in Gomati river and bedding material of laboratory animals.

CONCLUSION

The *E. coli* strains causing diarrhoea in different laboratory animal species were characterized using RAPD primers. There are very few variation in *E. coli* strains among the animal species suffering from diarrhoea has been found. Identification of pathogenic *E. coli* strains causing diarrhoea in laboratory animal species was observed by biochemical analysis of pathogenic E.coli. The primer designing are also required for quick identification of pathogenic gene of *E. coli* species in laboratory animals for further studies.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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