MOLECULAR DIFFERENTIATION OF *ESCHERICHIA COLI* O157:H7 AND *SHIGELLA* USING 16S RRNA PHYLOGENY

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Received: 31st October, 2022 **Accepted:** 3rd December, 2022 **Published:** 8th December, 2022

ABSTRACT

Background: Differentiation of *E. coli* O157:H7 from *Shigella* is reported to be difficult. Resource-poor settings rarely report on this or the percentage of *E. coli* O157:H7 that will cluster around *Shigella* in a phylogenic tree.

Aim: This study aimed to differentiate between *E. coli* O157:H7 and *Shigella* using 16S rRNA phylogeny.

Method: A total of 7 non-sorbitol fermenting *E. coli* of which 5 have been confirmed serologically as *E. coli* O157:H7, were used for this study. Amplification of hypervariable region of the 16S rRNA in these isolates followed by phylogenic analysis of the sequences was carried out for all the isolates. **Results:** Only one isolate clustered around *Shigella boydii* while the others clustered around *E.coli.* Twenty percent of the *E. coli* O157:H7 clustered around *Shigella.*

Conclusion: The use of 16S rRNA appears to be a good and veritable tool for differentiating the two genera.

Keywords: Differentiation; *Escherichia coli* O157:H7; *Shigella;* 16S rRNA; Phylogeny.

INTRODUCTION

Diarrhoea is a prevalent disease in both developing and industrialized nations (Devanga Ragupathi *et al.*, 2018). *Escherichia coli* and *Shigellae* are among the aetiologic agents of diarrhoea Both organisms were once thought to be separate (Goodridge, 2013) . However, the advent of *E. coli* O157: H7 which causes similar infection like *Shigella* and share a number of traits such as virulence mechanisms (Fukushima *et al.*, 2002), has necessitated the need to differentiate these pathogens as this will aid treatment of infections. Biochemical differentiation of *Shigella* and *E. coli* strains is typically challenging. *Shigella* species are considered metabolically inactive *E. coli* biogroups (Lan *et al.*, 2004). Enterohaemorrhagic *E. coli*, including *E. coli* O157:H7, are thought to be *Shigella* disguised as *E. coli* antigens. (Johnson, 2000) and based on DNA homology, Goodridge (2013) believed *Shigella* and *E. coli* to be a single species.

In resource-poor settings, technologies to efficiently differentiate between *E. coli* O157: H7 and *Shigella* are lacking. Although Brenner *et al.* (1972) found that *Shigella* and *E. coli* had a nucleotide similarity of 80 to 90%, the number (percentage) of *E. coli* O157: H7 that clustered around *Shigellae* in phylogentic analysis has not been reported in our environment.

Citation: Omoregie, Elemuwa, C. O., Isibor, J. O., Inyang, N.J., Elemuwa,G.U and Omoregie, R. (2022): Molecular Differentiation Of *Escherichia Coli* O157:H7 And *Shigella* Using 16s Rrna Phylogeny *BJMLS 7(2): 60 - 67*

Against this background, this study aimed to determine the number (percentage) of *E. coli* O157: H7 that will cluster around *Shigella* phylogenetically.

MATERIALS AND METHODS

Study area

The study was carried out in Central Hospital, Benin City (Oredo local government area), located on latitude 6.3298^0N and longitude 5.6225^0E. Oredo LGA has a population of 374,515 (National Population Commission, 2006). Central Hospital is a government-owned secondary level hospital that serves the health needs of people in Benin City as well as other LGAs in Edo State as it attends to referral cases from primary health care centres.

Study population

A total of 420 patients with gastrointestinal complaints attending Central Hospital, Benin City, Nigeria, were recruited for this study. Informed consent was obtained from all subjects or their parents/guardians in case of children prior to specimen collection. Approval for the study was given by the Ethical Committee of the Edo State Ministry of Health, Benin City.

E. coli O157: H7 isolates

A total of 7 sorbitol non-fermenting *E. coli* (from a previous study) were used for this study. Five had previously been serologically confirmed as *E. coli* O157: H7.

DNA extraction

DNA was extracted from the non-sorbitol fermenting bacterial isolates using the ZR Bacterial DNA extraction kit (Zymo Research Corporation, USA) following the manufacturer's instruction. Briefly, overnight cultures of the isolates on blood agar were emulsified in 200µL of molecular grade water inside a ZR Bashing Bead™ Lysis Tube. To this mixture was added 750µL lysis solution and the tube was centrifuged in a micro-centrifuge at 10000g for 1 min. After centrifugation, 400µL of the supernatant was placed inside a Zymo-Spin™ IV Spin Filter in a Collection Tube and centrifuged at 7000 rpm for 1 min. To the filtrate after centrifugation, was added 1200µL of Fungal/Bacterial DNA Binding Buffer. This was mixed and 800µL of the mixture was transferred to a Zymo-Spin™ IIC Column in a Collection Tube, and centrifuged at 10,000 x g for 1 minute. After centrifugation, the flow through was discarded and the remaining 800µL containing the DNA binding buffer was added to the zymo-spin™ IIC column in a collection tube and centrifuged at 10,000 x g for 1 min. Two hundred micro liters of DNA Pre-wash buffer were added to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute. This was followed by the addition of 500µL of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000 x g for 1 min. The Zymo-spin™ IIC column was transferred to a clean 1.5mL micro centrifuge tube and 100µL of DNA elution buffer was added directly to the column matrix, and centrifuged at 10, 000 x g for 30s. The eluate contains bacterial DNA which serves as template for polymerase chain reaction amplification.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) targeted at the 254bp V4 region of the 16S rRNA gene was performed using the extracted DNA as template and an in-house universal primer. The universal primer 27F- 5' AGAGTTTGATCMTGGCTCAG-3' and 1492R- 5' TACGGYTACCTTGTTACGACTT-3' was used. The reaction mixture contained PCR master mix, template DNA, primers and nuclease-free water in a final volume of 25µL. The PCR conditions were: a first initial denaturation of 94^0C for 3 mins (1 cycle), followed by another denaturation: 94^0C for 30s, which was followed by annealing at 54^0C for 30s and extension at 72^0C for 1 min. This was done for 35 cycles, and then followed by a final extension at 72^0C for 7 mins. The PCR mixture was held at 4^0C until needed for further analysis.

The agarose gel was placed in the electrophoresis tank. To the first well, 10µL of 50bp to 10000bp fast DNA ladder (New England Bio Labs Inc., England) mixed in loading dye was placed. The PCR product (10µL) for each non-sorbitol fermenting *E. coli* isolate was placed alongside with 2µL loading dye in other labeled wells of the agarose gel. A 90-volt current was passed through the gel for it to run for 60 minutes. After 60 minutes, the gel was viewed under UV trans-illumination and photographed with the aid of a computer program.

Sequencing

Each PCR product was cleaned with ExoSap mixture. Briefly, the ExoSap mixture and the PCR product were mixed in a ratio of 1:4 in a test tube and the test tube was incubated at 37^0C for 30 mins. The reaction was stopped by heating the mixture at 95^0C for 5mins. Sequencing reaction was done utilizing ABI Big dye V3.1 kit and the products were cleaned using the Zymo research sequencing cleanup kit (Zymo Research, USA). ABI 3500XL (ABI, USA) was used to analyze the sequencing reactions. Sequences data generated were analyzed with Geneious version 9.0.5 and phylogenetic trees were constructed using neighbor joining.

RESULTS

Phylogenetic analysis of 16S rRNA confirmed all (Figures 1, 2, 3, 4, 5, 6 and 7) but one isolate (Fig 5) as *E. coli*. The isolate not confirmed as *E. coli* clustered around a control strain of *Shigella boydii* (Fig 5).

DISCUSSION

Molecular analysis of 16S rRNA along with phylogenetic analysis confirmed the identity of the non-sorbitol fermenting *E. coli* as *E. coli* with the exception of one (isolate 40) which clustered around *Shigella boydii* ATCC 49812 (Fig V). Interestingly, isolate 40 reacted positively with *E. coli* O157:H7 antiserum. Many *Shigellae* have been reported to cross react with *E. coli* serologically, and vice versa (Liu *et al*., 2008; Fakruddin *et al*., 2015). It has been suggested that enterohaemorrhagic *E. coli* such as *E. coli* O157:H7 are essentially *Shigella* in a cloak of *E. coli* antigens (Johnson, 2000). Indeed, some authors have referred to both as one species and *S. dysenteriae* types 1, 8 and 10, *S. boydii* 13 and *S. sonnei,* are reported as isolated clones within *E. coli* (Liu *et al*., 2008).

DNA-DNA re-association studies showed that Shigellae exhibit >75% nucleotide similarity with *E. coli* (Brenner *et al*., 1972) and there are similarities between EHEC and *Shigella* species with respect to clinical behavior, virulence mechanisms and phylogenic background (Johnson, 2000). This may explain why isolate 40 clustered closer to *Shigella boydii* ATCC 49812 in the phylogenetic tree. Johnson (2000) stated that valid comparisons between *Shigella* species and *E. coli* require an understanding that *Shigellae* are actually pathotypes of *E. coli.* This may explain why isolate 40 clustered around *Shigella boydii* and means that it is actually an *E. coli* isolate.

Only one (14.29% and 20%) out of the 7 non-sorbitol fermenting *E. coli*, and 5 *E. coli* O157:H7 respectively, clustered around *Shigella* phylogenetically. This means that 80% of *E. coli* O157:H7 will be correctly identified using 16SrRNA phylogeny. This finding is at variance with previous reports in which 16SrRNA phylogeny was reported not to be effective in differentiating *E. coli* from *Shigella* (Fukushima *et al*., 2002; DevangaRagupathi *et al*., 2018).Use of *gryB* gene sequence followed by phylogeny, and whole genome sequencing has been reported to give better differentiation between *E.coli* and *Shigella* (Fukushima *et al*., 2002; DevangaRagupathi *et al*., 2018).None of these techniques were used in this study. This is a limitation of this study coupled with the fact that very few isolates were used.

In conclusion, the study revealed that 80% of *E. coli* O157:H7 can be effectively differentiated from *Shigella* using 16S rRNA phylogeny.

Therefore, molecular analysis and particularly phylogenetic study has demonstrated great potentials of differentiation, evidence of diversity and relatedness, hence, the use of 16S rRNA appears to be a veritable tool for differentiating the two genera.

The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate 155 has similar sequence with *Escherichia coli* strain K-15KW01 with accession number CP016358 (Figure 1).

The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate 127 has similar sequence with *Escherichia coli* strain CFSAN061772 with accession number CP042893 (Figure 2).

The phylogenetic tree was constructed by the Neighbor-Joining method program in the Generous package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate 140EC has similar sequence with *Escherichia coli* strain Ec40743 with accession number CP041919 (Figure 3)

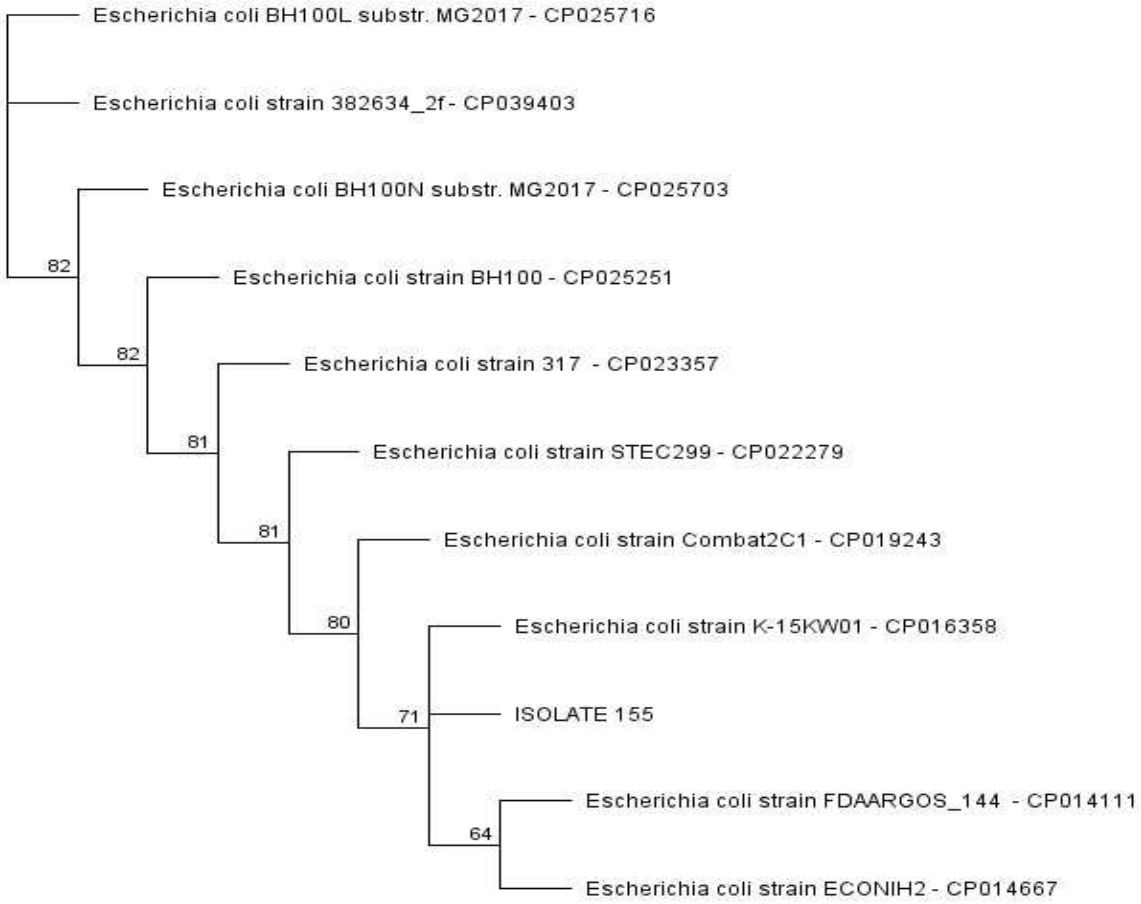

Figure 1: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria.

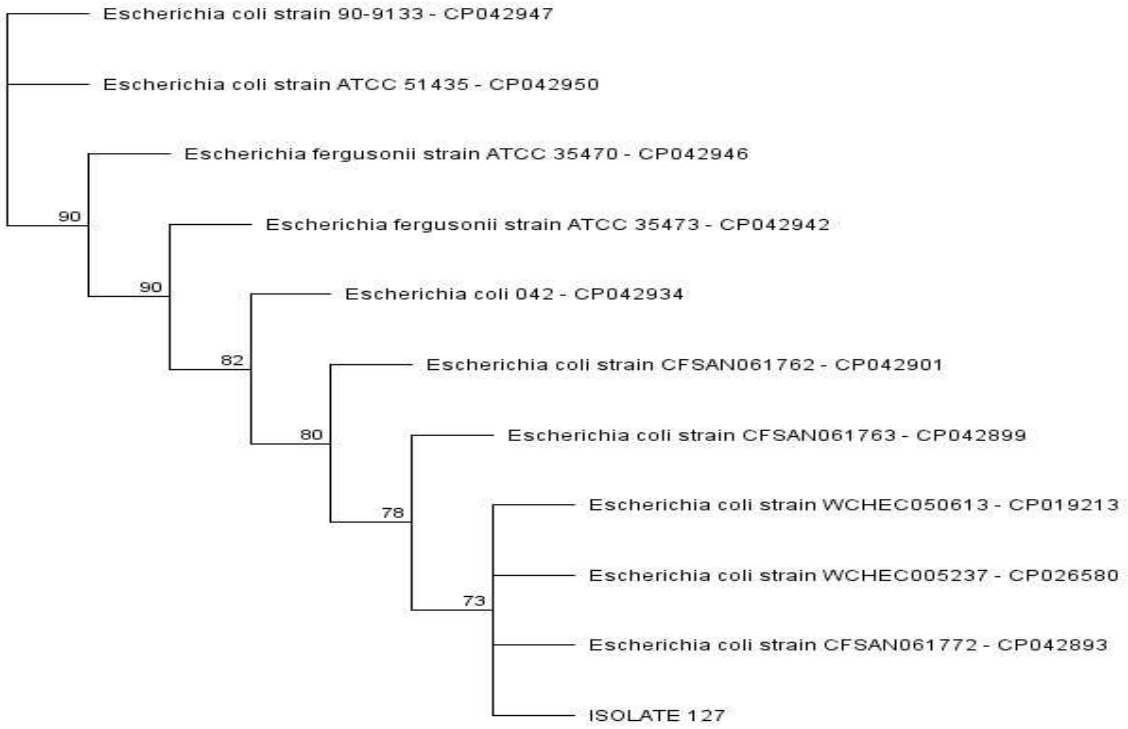

Figure 2: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria.

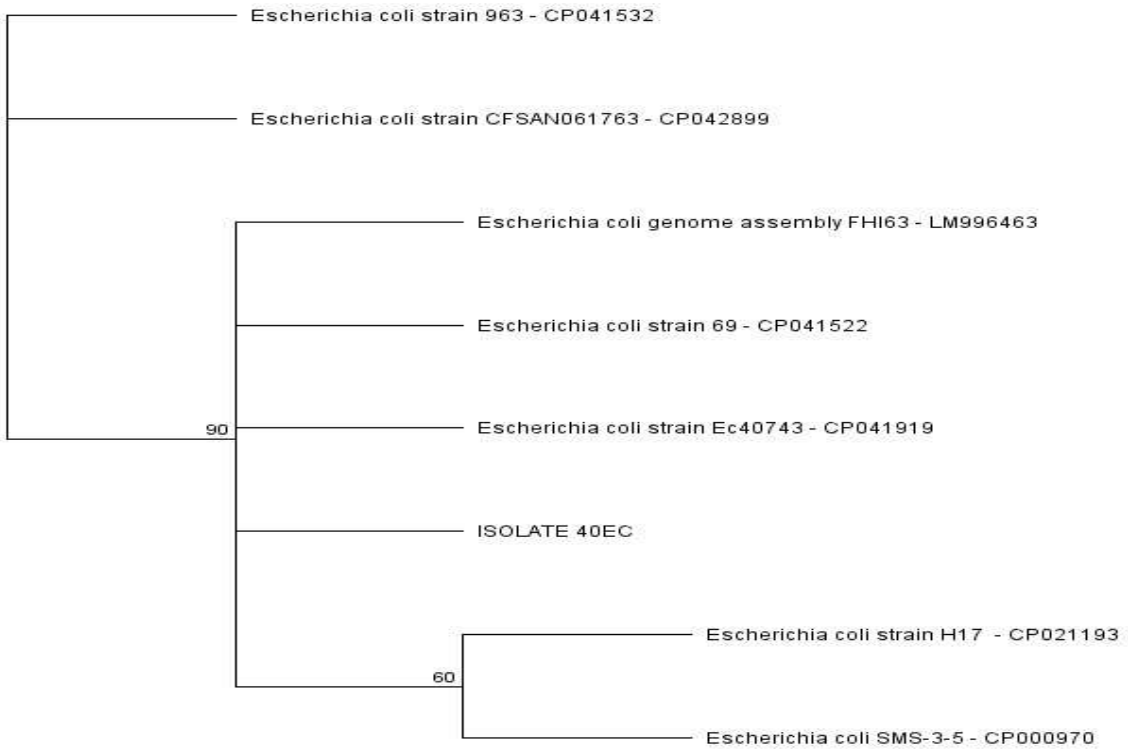

Figure 3: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria.

The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate 160A has similar sequence with *Escherichia coli* strain W2-5 with accession number CP032989 (Figure 4).

The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate 40 has similar sequence with *Shigella boydii* strain ATTCC 49812 with accession number CP026836 (Figure 5).

The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate 34 has similar sequence with *Escherichia coli* strain PYK20 with accession number MF582332 (Figure 6).

The phylogenetic tree was constructed by the Neighbor-Joining method programme in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate 353 has similar sequence with *Escherichia coli* strain PYK20 with accession number MF582332 (Figure 7).

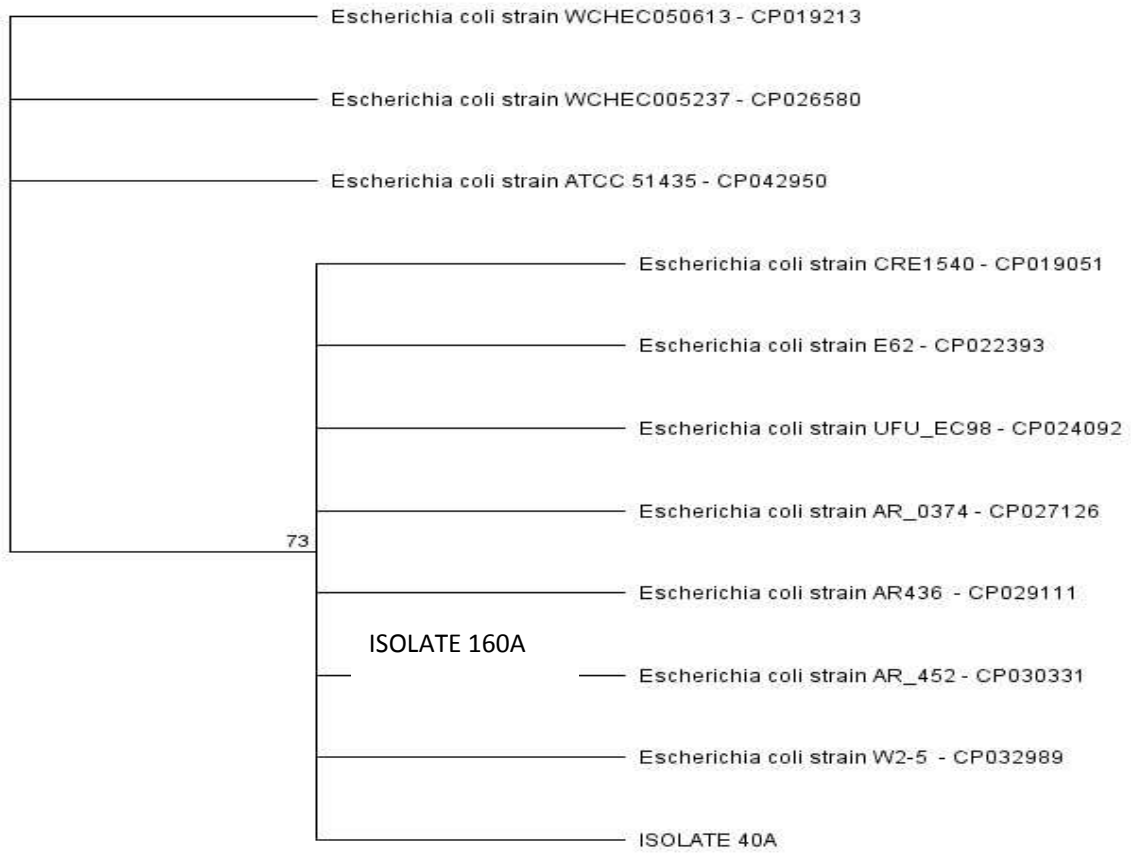

Figure 4: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria.

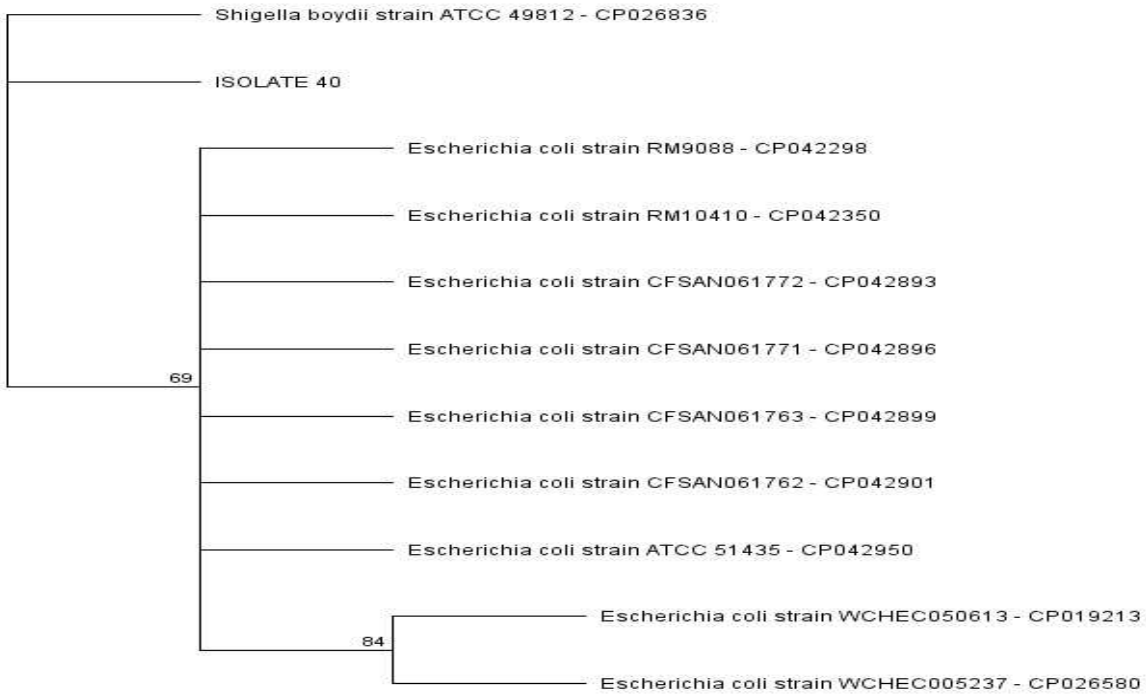

Figure 5: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria.

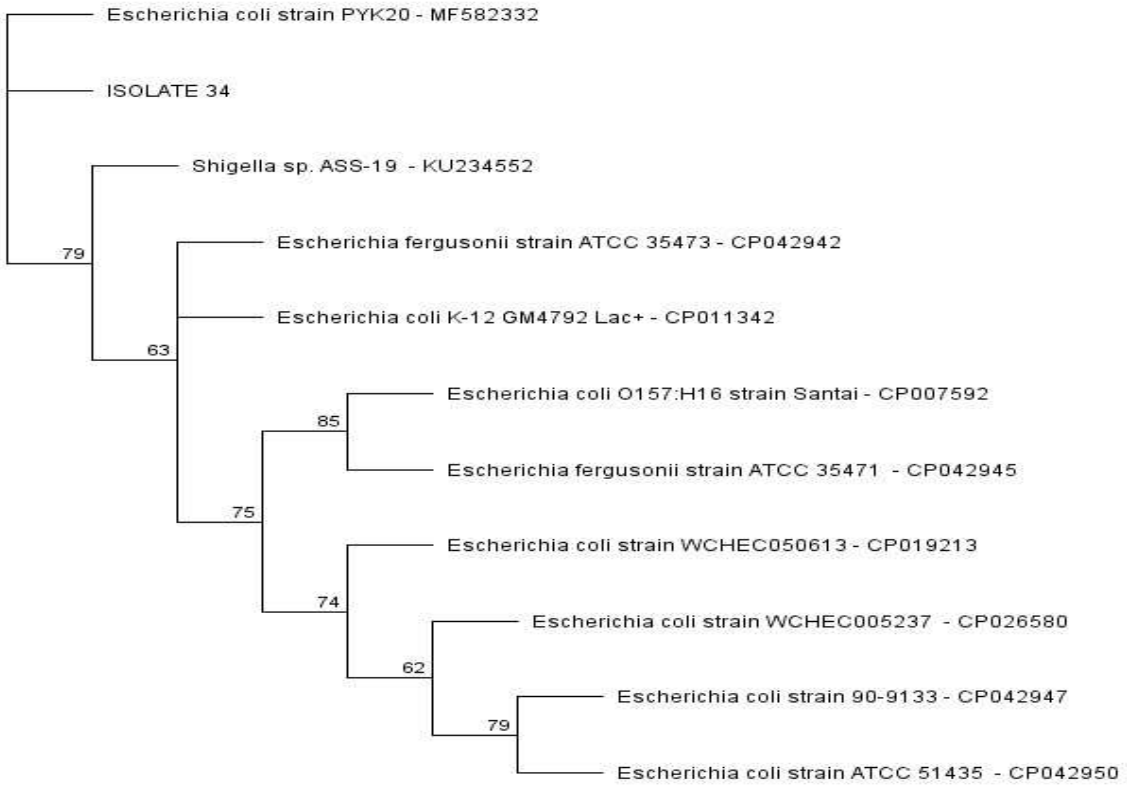

Figure 6: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria.

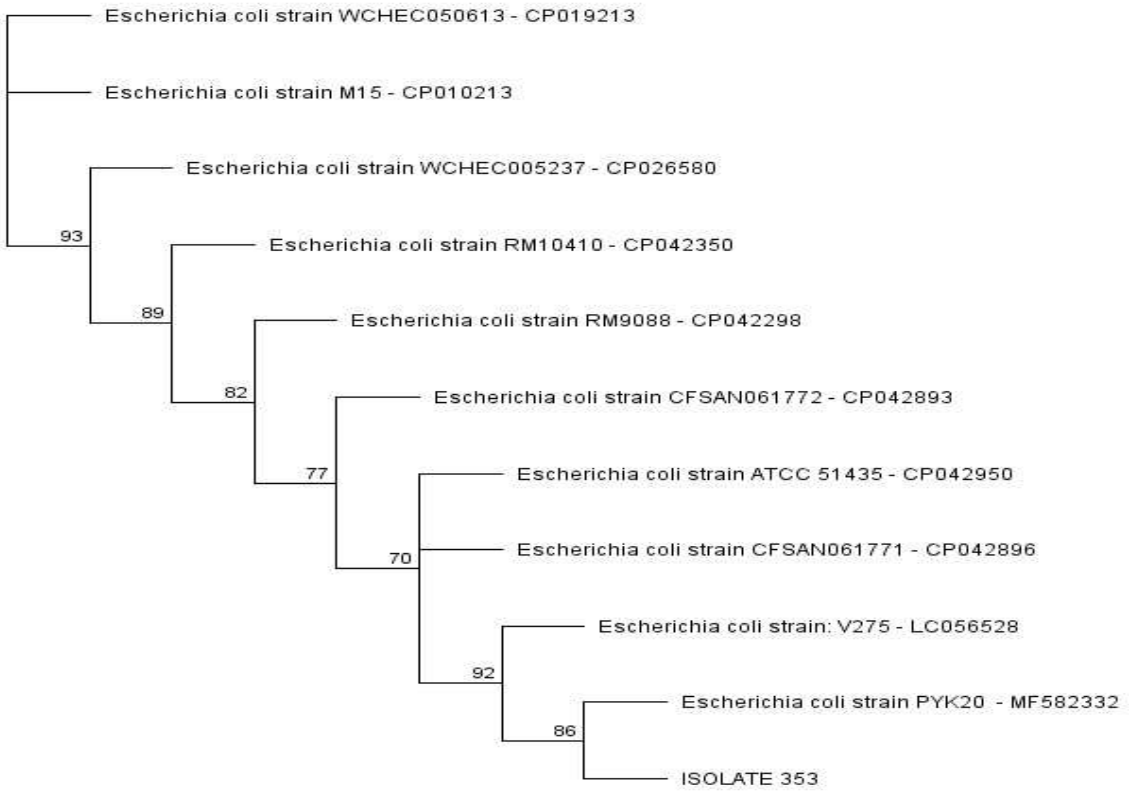

Figure7: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria.

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