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COMPLETE GENOME CHARACTERIZATION OF ST361 MULTI DRUG RESISTANT UROPATHOGENIC *ESCHERICHIA COLI* (UPEC) ISOLATED FROM CAPTIVE ASIATIC ELEPHANTS (*ELEPHUS MAXIMUS*)

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Abstract

Urinary tract infections (UTI) in large mammals like elephants are seldom reported though it can occur frequently in all age groups. *Escherichia coli* have been attributed to be one of the major causes of such infection and multiple resistances to antimicrobial agents may complicate therapeutic strategies for UTI. The aim of the study was to determine the whole genome sequences and its characterization of multi-drug resistant *Escherichia coli* isolated from urine of captive Asiatic elephant. The elephant was presented with history of dullness, dehydration with inappetance, hematuria, oliguria and was used for religious, entertainment and tourism purpose. The *Escheichia coli* isolate EDEC1 was phenotypically resistant to 23 different antimicrobials distributed across 11 different class and 32 resistant genes were identified including carbapenomase resistant gene through BLAST alignment of the predicted protein sequence with the Antibiotic Resistance Gene Database (ARDB). The whole Genome Sequencing (WGS) analysis revealed 537 different virulence factors including csgA, fimH, Gad, hlyE, Nlpl, terC, yehA, yehB, yehC, yehD. The EDEC1 isolate belonged to phylogroup A with Sequence Type (ST) 361 and CHtyper 99-54. The 16S rRNA gene based phylogeny revealed close relationship of the isolate with UPEC isolated from Red Panda, ATCC and Rodents.

Introduction

Antimicrobial resistance is recognized as one of the greatest threats to human health worldwide and ever increasing incidences of antimicrobial resistance in both domestic animals and human beings are well documented worldwide. Uropathogenic *Escherichia coli* (UPEC), one of the members of the extra-intestinal pathogenic *E. coli* (ExPEC) is a predominant pathogen causing urinary tract infections (UTIs) (Lee et al., 2014). These strains harbor a variety of virulence factors that allow them to establish an infection, including adhesins, toxins, host defense avoidance mechanisms and multiple iron acquisition systems (Kaper et al., 2004). Molecular characterizations of uropathogenic drug resistant *E coli* isolate from the urine of wild animals at

the whole genome level are limited. In this study, multidrug resistant uropathogenic *Escherichia coli* (UPEC) was isolated from the urine of captive Asiatic elephants (*Elephus maximus*) and whole genome sequence (WGS) of the isolate was analyzed to determine their genomic diversity and their pattern of antimicrobial resistance. Whole genome sequencing (WGS) of bacterial isolates is important to understand the transmission dynamics and antimicrobial resistance (AMR) gene diversity, now well placed to become a gold standard in bacterial typing and to determine relatedness between strains, as it can discriminate to characterize the complete genomic structure of isolates (Köser et al., 2014). Asiatic elephant, a schedule I species, under the Wildlife (Protection) Act, 1972, India, prohibits commercial trade and ownership of elephants are limited to those in possession of elephants before the law came into force. The Act was amended recently to insert a provision in Section 43(2) which carved out additional exceptions for the transfer and transport of captive elephants for '*religious and other purposes*' in addition to the special purposes mentioned under section 12 of the Act. In the present study, a multi drug resistant uropathogenic *Escherichia coli* isolated from captive Asiatic elephant is characterized by whole genome sequencing and phenotypical antimicrobial susceptibility test (AST).

Materials and Method

History and Sample Collection

A female Asiatic elephant aged ≥55 years used for religious and entertainment purposes was presented in the wild animal hospital of School of Wildlife Forensic and Health, NDVSU Jabalpur, India. The history indicated extensive travel of the elephant as companion animals for religious & rituals and kept outdoors. The elephant was feed by public in the open-markets, roads in addition to the diets provided by the caretaker but had limited access to regular routine nutritious diet and veterinary care. The elephant was dull, dehydrated with inappetence, oligouria and hematuria. The urine (pH 5.5) was positive for Glucose, ketone bodies, urobilinogen, bilirubin, nitrite and traces of protein with specific gravity of 1.045. The microscopic examination revealed pus cells (7-10/HPF), RBCs/crystals (8-9/HPF) and epithelial cells (12/HPF). At least 20ml of midstream voided urine was collected aseptically in sterile containers on 27-05-2023. Urine sample was stored at 4ºC for bacterial analysis.

Escherichia coli Isolation and Antimicrobial Sensitivity

Urine samples were inoculated in buffered peptone water and incubated at 37°C for 18-24 hrs. Pre-enriched culture from peptone water was inoculated in McConkey agar and incubated at 37°C for 18-24 hrs. The pink coloured colonies were further streaked on eosin methylene blue agar and incubated at 37°C for 18-24 hrs. The purified colonies on eosin methylene blue agar was confirmed in automated bacterial identification (BD PHOENIX^{TM} M50, Becton and Dickson, Franklin Lakes, NJ, USA) using both an oxidation-reduction indicator and turbidity growth detection to provide both rapid and accurate susceptibility results. Antimicrobial susceptibility test system was used to determine minimum inhibitory concentration (MIC) in µg/mL for 26 antibiotics (Table 1) in NMIC/ID panels except for Cefpodoxime (10µg/disc), Cefepime/Clavulanic acid (40µg/disc), Ampicillin/Sulbactam (20µg/disc), Enrofloxacin (5µg/disc), Doxycycline (1µg/disc) and Erythromycin E (15µg/disc) which were tested by disc

diffusion method following CLSI guidelines (CLSI, 2024). Antibiotic susceptibility results were interpreted according to the CLSI standard VET01 (CLSI, 2018).

Whole Genome Sequencing

Genomic DNA from *E. coli* cultures were extracted using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA from cultured *E. coli* was quantified with DNA HS assay kit following manufacturer's protocol using Qubit 4.0 flurometer and diluted to 0.2ng/µl and library prep performed using the Illumina trueseq Nano DNA Library kit protocol according to the manufactures instructions. Briefly, gDNA was fragment and tagged with adapter sequences using a transposase enzyme in a process known as tagmentation. Using a limited cycle PCR step, indices were introduced. This helped in de-multiplexing after sequencing was complete. A size selection clean-up was done using AMPure beads (AGENCOURT) and normalized to ensure equal representation of all libraries. The normalized libraries were pooled, denatured and loaded on the Illumina Novaseq 6000 platform using 2X150bp chemistry as per manufacturer's protocol. The annotation was done by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

Bioinformatic Analysis

The high quality reads obtained using Trimmomatic v0.38 (Bolger et al., 2014) to remove adopter sequences, ambiguous reads (reads with unknown nucleotide "N" more than 5%) and low quality sequences (reads with more than 10% quality threshold < 25 phred score), were aligned to the *E. coli* strain K-12 sub strain MG1655 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000005845.2/) as reference genome in BWA MEM 0.7.17 (Li and Richard, 2009). The consensus sequence, SNPs and InDels was extracted using SAMtools mpileup (v 0.1.18) (Li et al., 2009). The GC skew for both positive and negative strands, features, ORF and GC content were mapped by Proksee (Jason et al., 2023; https://proksee.ca). Resfinder (4.4.1) (https://cge.cbs.dtu.dk/services/ResFinder/) was used to identify acquired antimicrobial resistance genes from raw fastq files. Virulence finders 2.0 were used to screen the virulence genes of the isolate (Jolley et al., 2018). The Center for Genomic Epidemiology (CGE) database (http://www.genomicepidemiology.org/) was used for MLST profile and identification of the mobile genetic elements of the isolate. Phylogroup was assigned (A, B1, B2, C, D, E, and F) using the revised Clermont method (Accessesed on 1.4.2024) (Beghain et al., 2018). The 16S rRNA gene of few representative member of *Enterobacteriaceae* family were retrieved and phylogenetic relationship of the isolate was established using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The CHTyper, a web tool for Subtyping of Extra-intestinal Pathogenic *Escherichia coli* was used for subtyping the isolate (Roer et al., 2018) (Accessed on 14.12.2023). The evolutionary history was inferred from 16S rRNA gene of the isolates by Maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) in MEGA 6 (Tamura et al., 2013) with the bootstrap consensus tree of 1000 replicates (Felsenstein, 1985).

Results

Antibiotic Sensitivity Assay

The isolate was found to be phenotypically resistant against 23 antimicrobial agents of 11 antimicrobial classes and was susceptible to Amikacin, Chloramphenicol and Colistin (Table 1). The correlation of phenotypic resistance with genotypes resistance pattern of the isolate were The resistance against 13 antibiotics was observed both in both . The whole genome analysis revealed presence of 13 antimicrobial resistance gene from 5 classes of antibiotics along with hydrogen peroxide (Additional Table 1).

Whole Genome Sequencing

The Whole genome sequence of the sample yielded ~1.01Gb of high quality (HQ) data corresponding to 1,006,225,832 of HQ bases. There were 367 contig and N50 contig were 67939. The whole genome of the isolate was mapped to 87.99% with 94.95% genome coverage with reference to E. coli strain K-12 sub strain MG1655. The total length obtained in the whole genome sequencing was 4,641,652bp with 51.03% of GC content and scanned gaps of 366bp. The genome sequence of the EDEC1 isolate was submitted in public database (BioProject PRJNA1034305, Accession no. CP138910). The GC skew [(G − C)/(G + C)] plot showed an asymmetric compositions of G and C on two sides of the chromosome, suggesting the replication origin and terminus (Frank and Lobry,1999) (Fig1). A total of 30,667 SNP's were obtained with 26,887 SNPs within the genes while 3780 SNPs were found to be intergenic. The numbers of mapped InDels were 282 (73 genic and 209 intergenic InDels). The whole genome sequence revealed total genes of 4413 with 4,036 coding proteins (CDS). There were 117 RNA genes identified (rRNAs: 5S, 16S, 23S- 8, 7, 7 respectively, tRNAs: 86, ncRNAs:9) .

Typing and Virulence Factor

The clement analysis assigned the isolate in phylogroup A with TspE4: -, arpA: +, chu: -, yjaA: + profile. The EDEC1 isolate was in the same clade of uropathogenic *E coli* isolated from Red panda (*Ailurus fulgens*) and related with *Escherichia fergusonii* isolates from dolphin (*Tursiops truncatus*), *Escherichia marmotae* isolates from Marmota himalayana Rodents, poultry and human isolates in the phylogenetic tree (Fig 1). ML tree of the isolate The MLST (Achtman) profile of the isolate matched with sequence type (ST) 361 and the CH-typing was CH99-54. BLAST alignment with the Virulence Factor Database (VFDB) showed that the measured *E. coli* contained 537 virulence factors (Additional table 2), including outer membrane protein, flagella, P pili, S pili, fimH coding for type 1 fimbrial adhesin, type I pili, cytotoxic necrosis factor and hemolysin. The Hemolysin E (HlyE), a novel pore-forming toxin of *Escherichia coli*, was mapped on the genome (1229483-1230400) of EDEC1 (Additional table 3). The reference database revealed presence of 2 composite transposons and 17 insertion sequence (19 out of 85 in the database) from 6 different families in 5 groups (Additional table 4). The phylogentic relationship revealed that EDEC1 isolate was in the same clade of UPEC isolates from Red panda, ATCC 11775 reference organism isolated from a Danish patient's urine in 1941 and rodents along with isolates from dolphin, poultry and food (Fig 2).

Discussion

The emergence of multidrug resistance (MDR) *Escherichia coli* is a well-known global health concern and a common pathogen linked with community-associated nosocomical infections that possesses numerous resistance genes in its genome (Paterson, 2006). Whole genome Sequence (WGS) allows rapid and cost-effective acquisition of the data for the presence of specific antibiotic resistance genes, the determination of various isolate and plasmid replicon classification and typing markers & techniques, having significantly higher specificity resolution and typing markers and essential for control and prevention strategies to combat the growing threat of AMR and the implementation of multifaceted interventions (Shelenkov, 2021). Analysis of UPEC genomes and the comparison with the *E. coli* genomic database is important to reveal the plasticity of UPEC pan genome and the presence of UPEC-specific PAIs genes to encode putative virulence factors, such as pilus proteins, adhesins, and iron-uptake systems.

The EDEC1 isolate was resistant to all β-lactams (Table 1). BLAST alignment of the predicted protein sequence with the Antibiotic Resistance Gene Database (ARDB) showed that the strain contained 32 resistance genes including carbapenemase encoding genes, namely $bla_{\text{NDM-5,}}$ class B metallo ß-lactamase (MBL) and a series of additional β-lactamase genes, $bla_{\text{CMY-145}}$ encoding, an AmpC-type cephalosporinase and *bla*OXA-1 encoding a narrow-spectrum class D ß-lactamase, oxacillinase, *blaTEM-1B*, a narrow-spectrum penicillinase, *bla*TEM gene. Other detected resistant determinants in NDM-5 producing EDEC1, included *dfrA*12, *aadA* 12 which accounted for high-level resistance against aminoglycoside and trimethoprim. The EDEC1 isolate was susceptible to Colistin, Chloramphenicol and Amikacin, similar to susceptibility profiles observed in ST361 *E coli* isolated in a Tertiary Hospital in South Korea (Park et al., 2020).

The virulence factors of *Escherichia coli* can broadly be divided into two groups: bacterial cell surface and secreted virulence factor (Shah et al., 2019). The isolate harbored 537 virulence factors (Appendix 1). Among the bacterial cell surface virulence factors, different components of type 1 fimbriae, a critical virulence factor in UPEC-derived UTIs in humans, fimA gene, a major subunit (4543115-4543663), fimB gene, regulatory protein (4540957-4541559), fimE gene, regulatory protein (4542037-4542633), fimI, minor subunits/adaptor subunits gene (4543728-4544267), fimC gene, a chaperone (4544304-4545029), fimD gene, an usher (4545096-4547732), fimF gene minor subunits/adaptor subunits (4547742-4548272), fimH gene, D-mannose specific adhesion (4548808-4549710) have been mapped in the genome sequence of EDEC1 isolate (CP138910). The gene fimH is the most common adhesion gene observed in uropathogenic *E. coli* isolates (Dadi et al., 2020). Uropathogenic *E. coli* (UPEC) attaches to the uroepithelium through type 1 pili, which bind the receptors uroplakin Ia and IIIa that mediate invasion and apoptosis (Croxen and Finlay, 2010). The expression type 1 fimbrae accounts for less urine output in UTI of human being and responsible for bioflim formation in mammalian bladders by Gram-negative uropathogens along with genes responsible for metabolism of exopolysaccharides like cellulose (bcsA, bcsB, bcsC, bcsD, bcsE, bcsF, bcs, bcsZ genes) (Zhou et al., 2023). An important outer membrane protein *omp*A gene, responsible for intracellular survival and evasion from the body's response has been mapped in the isolate. Among the secreted virulence factors, Hemolysin genes, encoded by the *hlyCABD* operon, a prototypical calcium-dependent repeats in toxin secreted

protein that can penetrate uroepithelial cell membranes (ybhG, hlyE, ybeX) have been annotated. The gene fhuF (4604875-4605663) coding for ferric sidophore reductase for Iron acquisition, also an important marker for bacterial secretory virulence factor (Behnoush et al., 2021) was identified in the UPEC isolate from captive Asiatic elephants. The presence of iron uptake system increases the pathogenicity, contributing to the bacterial survival through heme and ferritin (Braun, 2001). *E. coli* strains responsible for extra-intestinal infection are far more likely to be members of phylogroups B2 or D than A or B1 (Dadi et al., 2020). However, the EDEC1 isolate was a member of phylogroup A as reveled by Clemant analysis where as the UPEC isolate from Red panda (CP063214.1) was a member of B2 with profile of TspE4: +, arpA: -, chu: +,yjaA: +. Massella et al., (2020) observed that the most common phylogroup of E coli samples isolated from Companion Animals, Livestock, Wildlife, Humans and Food was B1, followed by A, D and B2. The phylogroup A was the most represented (≥40%) in dairy, fishery, poultry and swine sources. The phylogenetic relationship of EDEC1 isolate revealed that the isolate was in the same clade as that uropathopathogenic *E coli* isolated from Red panda and *Escherichia coli* strain NCTC 9001, a strain isolated from urine and used for aerosol detection, media testing and quality control testing. Transposons, intergreons, plasmids plays major role in spread of resistance in uropathogenic *Escherichia coli* (UPEC) pathotype (Rozwadowski and Gawel, 2022). In the EDEC1 isolate, 17 insertion sequences were identified (Supplementary table). There are no specific MGE associated with UPEC isolates but the profile of MGE in uropathogenic isolate from any wild animals is not reported earlier.

Captive elephants kept as companion elephants are often taken to rivers, ponds for drinking water, bathing and takes part in religious and tourist activities. The spread of resistant bacteria from such elephants are very high as they are always in close proximity to human beings and share the same ecosystem as human. Understanding the transmission of drug resistant isolates in captive elephnats is crucial in the context of *One Health* approach due to its impact on public and animal health. Novel high-risk carbapenem resistant *E. coli* (CREC) is continuously emerging worldwide and such bacterial clones had been isolated from fresh water fish (Midwives et al., 2023). ST361 clone has been recently reported as the most frequent lineage of NDM-5 producing *E. coli* from many parts of the world across many species (Haeili et al., 2023; Tsilipounidaki et al., 2022). The present study is the first report of characterization of Uropathogenic Carbapenem resistant *E. coli* carrying NDM-5 in Asiatic elephants. Previously, similar Uropathogenic *E. coli* bacteria was reported in captive red panda (*Ailurus fulgens*). The spread of UPEC in different species of captive wild animals is a matter of concern for in situ conservation and further epidemiological study for better understanding of the antibiotic susceptibility of *E. coli* strains isolated wild animals is warranted. It has been described that NDM-producing strains are frequently resistant to a wide range of antimicrobial agents, due to co-harboring additional resistance determinants and NDM-5 appears to be more confined to *E. coli* isolates (European Centre for Disease Prevention and Control, 2023). Though antibiotic usage in veterinary medicines, livestock management, and agricultural practices has been attributed to the emergence of resistance among the strains from animal and

environmental origin (Manyi-Loh *et al.*, 2018), such community based transmission are also possible where captive wild elephant had no history of antibiotic administration.

The captive wild animals which share common habitat with human beings have the potential to spread anti microbial resistant genes to human-associated bacterial species, domestic animals and environment. The movement of such wild animals as elephants should be restricted, screened for their health and presence of AMR bacterial species before moving from one city to another. Use of antibiotics on such animals should be monitored and community acquired cases on such animals as elephants should be characterized in detail. The wastes from such wild animals should be disposed only after following proper waste disposal protocol strictly.

Declaration of Competing Interest

The authors declare that there is competing interest in any manner with respect to research work of the manuscript.

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Table 1: Antibiogram pattern of *E.coli* isolated from urine of captive Asiatic elephants. *Zone of Inhibition-[1, 2, 3,4, 6] No zones of inhibition were observed; [5] -10 mm

Sl.No	Class	Antibiotics	MIC (µg/ml)/ Zone of inhibition (mm)	Result	Genotypes of resistance genes
1	Aminoglycoside	Amikacin	≤8	Susceptible	
		Gentamicin	>8	Resistant	mph(A) (U36578, D16251)
2	Carbapenem	Imipenem	>8	Resistant	blaNDM-5 (JN104597)
		Meropenem	8	Resistant	blaNDM-5 (JN104597)
3	Cephalosporin	Cefazolin	>16	Resistant	C-ampC (KR010384)
		Cefoxitin	>16	Resistant	blaCMY-145 (KX470426), blaNDM-5 (JN104597)
		Ceftazidime	>16	Resistant	blaCMY-145 (KX470426), blaNDM-5 (JN104597)
		Cefotaxime	>32	Resistant	blaCMY-145 (KX470426),

					blaNDM-5 (JN104597)
		Cefepime	>16	Resistant	blaNDM-5 (JN104597), blaOXA-1 (HQ170510)
		*Cefpodoxime[1]	0 mm	Resistant	blaNDM-5 (JN104597)
		*Cefepime/ Clavulanic acid[2]	0 mm	Resistant	blaNDM-5 (JN104597), blaOXA-1 (HQ170510)
4	Monobactam	Aztreonam	>16	Resistant	*FtsI* (PBP3) NC_000913.3
5	Penicillin	Ampicillin	>16	Resistant	blaCMY-145 (KX470426), blaTEM-1B (AY458016), blaNDM-5 (JN104597), blaOXA-1 (HQ170510)
		Piperacillin	>64	Resistant	blaCMY-145 (KX470426), blaNDM-5 (JN104597)
		Amoxicillin-Clavulanate	>16/8	Resistant	blaCMY-145 (KX470426), blaNDM-5 (JN104597), blaOXA-1 (HQ170510)
		Piperacillin-Tazobactam	>64/4	Resistant	blaCMY-145 (KX470426), blaNDM-5 (JN104597), blaOXA-1 (HQ170510)
		*Ampicillin/ Sulbactam[3]	0 mm	Resistant	blaCMY-145 (KX470426), blaTEM-1B (AY458016), blaNDM-5 (JN104597), blaOXA-1 (HQ170510)

6	Phenicol	Chloramphenicol	8	Susceptible	
7	Fluoroquinolones	Ciprofloxacin	>2	Resistant	gyrA (p.S83L)
		Levofloxacin	>4	Resistant	gyrA (p.S83L)
		*Enrofloxacin[4]	0 mm	Resistant	gyrA (p.S83L)
8	Diaminopyrimidine-sulfonamide	Trimethoprim-Sulfamethoxazole	>2/38	Resistant	dfrA12 (AM040708), sul1 (sul1_U12338)
9	Tetracycline	Tetracycline	>8	Resistant	tet(B) (AF326777), tet(A) (AF534183)
		*Doxycycline[5]	10 mm	Resistant	tet(B) (AF326777), tet(A) (AF534183)
10	Polymyxin	Colistin	≤0.5	Susceptible	
11	Macrolide	*Erythromycin[6]	0 mm	Resistant	mph(A) (U36578), mph(A) (D16251)

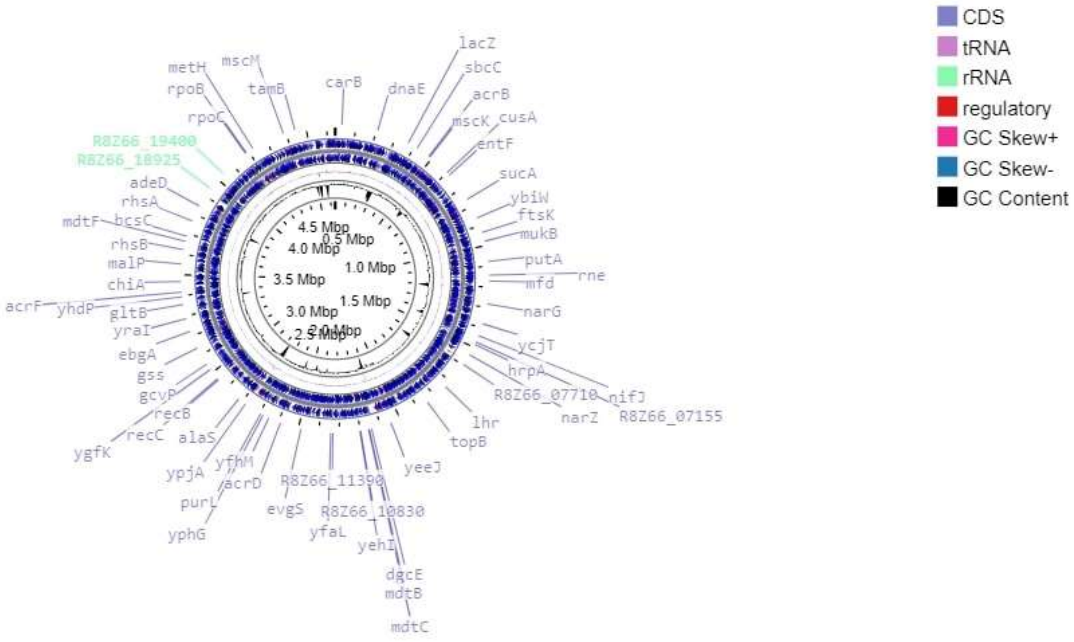

Figure 1: Circular map of EDEC1 (CP138910) showing the different features of the genome

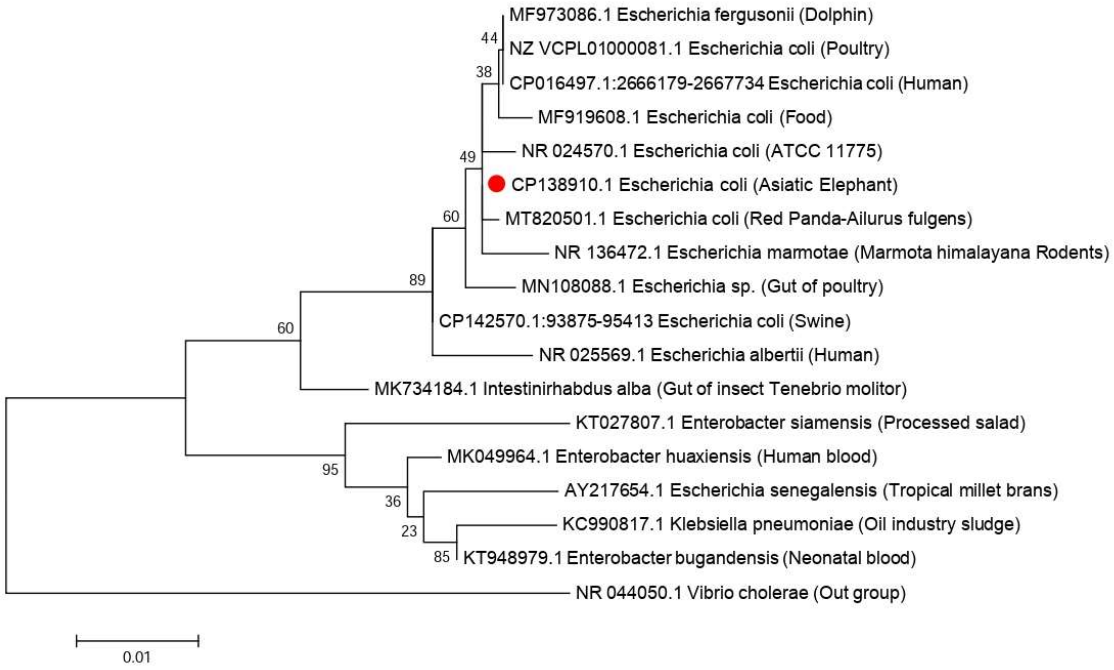

Figure 2: Phylogenetic relationship of the isolated strain and closely related species using the Maximum likelihood method. Bootstrap values (expressed as a percentage of 1000 replications) are given at the branching points. Vibrio albensis strain RC782 (NR_044050) was used as out group.

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