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Research Article

Molecular Characterisation of *Enterobacter* Species Associated with Community-Acquired Urinary Tract Infection

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A B S T R A C T

Background: The most typical diseases brought on by *Enterobacter*ales are urinary tract infections (UTIs), which include community-acquired and hospital-associated infections. Numerous such infections are caused by the *Enterobacter* species, which is a member of ESKAP pathogens known for antibiotic resistance. The current study aims to accurately characterise *Enterobacter* spp., implicated in community-acquired UTIs, by genetic methods.

Patients and Methods: A total of 50 midstream urine specimens were collected from patients with uncomplicated UTIs. Specimen collection was conducted from August to September 2022. The samples were cultured on selective culture media under aerobic conditions for 24 hours at 37 °C. The isolates were primarily identified by standard bacteriological methods. The molecular study included bacterial genomic DNA extraction and the use of specific primers for the amplification of 465 bp of *16S rRNA* gene, and sequencing along with the construction of a phylogenetic tree.

Results: Out of 50 urine specimens, only 7 gave positive culture results for *Enterobacter* spp. (14%), encompasses *Enterobacter* spp. 4 (57.1%) and *Enterobacter cloacae* 3 (42.8%). These isolates were confirmed by PCR for *16S rRNA* gene and sequencing.

Conclusion: This *16S rRNA*–based inclusive tree has provided an extremely inclusive tool about the high ability of such genetic fragments to efficiently identify *Enterobacter* spp. using this genetic fragment.

Keywords: *Enterobacter* spp., Urinary Tract Infection, *16S rRNA* Gene, Community Acquired UTI

Introduction

Urinary tract infections (UTIs) represent an expensive and common public health problem worldwide due to their high prevalence, the difficulties associated with their management, and their complications.[1] Approximately every year more than 200 million people are diagnosed with UTIs all over the world.[2]

UTI is one of the most common bacterial infections in humans in terms of community or hospital-acquired infections. A higher number of Enterobacterales, such as *Enterobacter* spp. or *Klebsiella* spp., are usually considered to be more frequently involved in hospital-acquired infections and more frequently responsible for complicated UTIs,[3] whereas most community-onset uncomplicated UTIs (uUTIs) are caused by members of the Enterobacterales family, including *Escherichia coli* and *Proteus mirabilis.*

Enterobacter is a genus belonging to the family of Enterobacteriaceae that is associated primarily with healthcare-related infections. There are currently 22 species of *Enterobacter*. However, not all species are known to cause human disease. *Enterobacter* species are responsible for causing many nosocomial infections and, less commonly, for community-acquired infections, including UTIs and respiratory infections. Certain species of this bacterium can be a part of the microflora of the mammalian gastrointestinal tract.[5]

Enterobacter species belong to the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species), which is mainly responsible for resistant nosocomial infections.[6] *Enterobacter cloacae* complex includes seven species. All these species are genotypically very close, with more than 60% DNA-DNA homology. The main species is *E. clocaea*. Numerous investigations have established that *E. cloacae* colonisations and infections are caused by the dispersal of multiple clusters that correspond to well-known major multilocus sequence types and that there is no connection to the geographic source of the organism.[7]

The virulence of this bacterium depends on a variety of factors. Like other gram-negative enteric bacilli, the bacteria use adhesins to bind to host cells. The presence of a lipopolysaccharide (LPS) capsule can aid the bacteria in avoiding opsonophagocytosis. The LPS capsule can initiate a cascade of inflammation in the host cell and may further lead to sepsis.[4] In gram-negative bacteria, the type III secretion system (TTSS) is recognised as a pathogenicity factor, and the *E. cloacae* isolated from clinical infections have been found to possess this factor.[8]

The accurate identification of species and subspecies remains a challenge. The development of genome sequencing has rapidly modified the phylogeny of the genus, particularly that of the *E. cloacae* complex.[9]

Multilocus Sequence Analysis (MLSA) of housekeeping genes, in part, and sequencing of the *16S rRNA* have recently allowed the characterisation of new *Enterobacter* species isolated from human infections.[10]

The current study aims to investigate the involvement of *Enterobacter* spp. in community-acquired urinary tract infections through the molecular characterisation of these bacteria.

Methodology

Study Setting and Duration

The study was designed as a cross-sectional study. It was conducted in the Faculty of Science for Women at the University of Babylon. The specimens were collected between August and September 2022 from patients who attended the Al-Hilla General Teaching Hospital and Imam Sadiq Teaching Hospital.

Patients and Specimen Collection

The study population comprised 50 patients with UTIs whose ages ranged between 20 and 61 years. The specimen was midstream urine collected by clean-catch method, using sterile, screw-capped plastic, and transported using a cool box to the microbiology laboratory in the College of Science for Women. Haematuria, pyuria, or phosphate crystal deposits (cloudy urine) were the first signs that could be detected in the urinalysis. The exclusion cases included patients with prostate disease and those who had undergone catheterisation within the previous two weeks.

Isolation and Identification of Bacteria

The urine samples were inoculated onto blood agar and MacConkey agar. For 24 hours, cultures were incubated at 37 °C in an aerobic environment. The clinical isolates were primarily distinguished on the basis of colony characteristics such as margin nature, translucence, haemolysis presence, colour, and odour, while the gram stain was set for identification: gram-positive or negative. Additionally, the microscopic examination was done to determine the shape of the organism cells such as rod, cocci, pleomorphic, spinal, etc.

Ethical Approval

Every patient was allowed to be enrolled as a participant in the study only after written informed consent about name, medical history, intake treatment, age, and type of specimen. The condition for granting ethical approval was that the medical consultant who provided medical coverage also gave permission.

Genotyping Assay

The company's instructions (Promega, USA) regarding bacterial DNA extraction were followed. DNA was extracted

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for 20 *Enterobacter* spp. isolates. The concentration of DNA was spectrophotometrically determined by evaluating its optical density at 260 nm. The purity of the DNA solution was indicated by a ratio of OD260/OD280, which is in the range of 1.8 ± 0.2 for pure DNA.[11]

The primers were rehydrated according to the instructions of the producing company (Alpha-USA) by using deionised nucleases free distilled water to obtain 1000 pg concentration as stock solution of primers. The forward primer sequence was 357F (5-CTACGGGGGGCAGCAG-3), and the reverse primer sequence, 806 R (GGACTACCGGGGTATCT).[12] They were designed to target the amplification of genomic sequences (465 bp) of *16S rRNA* loci.

The DNA amplification reaction was carried out in 25 µL volume of reaction mixture containing 5 µL DNA, 12.5 µL GoTaq® Green Master Mix (Promega), 2.5 µL forward primer, 2.5 mL reverse primer (10 pMol), and 7.5 µL nuclease-free water.

The PCR amplification programme was done in a PCR thermocycler. The initial denaturation was done at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 61 °C for 1 min, and elongation at 72 °C. The amplification was completed with a final extension at 72 °C for 10 min. The PCR product was tested using agarose gel electrophoresis technique at a concentration of 1.5% (w/v) of agarose, with the addition of ethidium bromide (0.5 µg/mL). As a molecular size marker, selecting a 100-bp ladder (Bioneer, South Korea), a solution of DNA molecules of different lengths used in agarose. All PCR fixed bands were checked for precision and consisted of only one pure and distinct band to be successfully subjected to sequencing.

DNA Sequencing of PCR Amplicons

The solved PCR amplicons were commercially sequenced from both (forward and reverse) terminals according to the instruction manuals of the sequencing company (Microgen Inc. Geumchen, Seoul, South Korea). The chromatographs obtained from the sequence folders of Applied Biosystems (ABI) were thoroughly examined, ensuring that the variances and annotation were not the result of PCR or false sequencing. The factual locations and other details of the restored PCR fractions were distinguished by comparing the observed DNA sequences of the examined samples with the restored neighbouring DNA sequences of the NCBI blast.

Explanation Sequencing Data

Using the BioEdit sequence alignment editor (version 7.1), the various sequencing findings of the PCR products were examined, aligned, edited, and as long as with the specific sequences in the indication database comparison with NCBI (DNASTAR, Madison, WI, USA). Each sequenced sample's observed variations were identified by the numbering of PCR amplicons and their location within the corresponding genome.

Comprehensive Phylogenetic Tree Construction

The blast results of the detected variants were merged and ranked using Clustal Omega-based techniques. Additionally, the analysis of the phylogenetic tree and the degree of genetic convergence between these isolates from type UPGMA tree. Subsequently utilising the IToL (Interactive Tree of Life) programme, the entire contained tree, incorporating the discovered difference, was shown as a polar clad gram (Letunic and Bork, 2019). Each set of graded phylogenetic categories for bacteria in the complete tree has appropriately coloured sequences.

Results

Prevalence Rate of *Enterobacter* spp.

Out of 50 urine specimens, seven *Enterobacter* spp. (14%) were isolated, which included 4 (57.1%) *Enterobacter* spp. and 3 (42.8%) *Enterobacter cloacae*. The primary diagnosis of these isolates depended on the colonial morphology exhibited on blood agar. After cultivation for 24 hours at 37 °C, large, white, smooth, and flat colonies with complete margins were observed without beta haemolysis. Additionally, these colonies were negative for oxidase and positive for catalase tests. The diagnoses of all of these isolates were confirmed by sequencing of *16S rRNA* loci of genomic DNA.

Molecular Study

Gel Electrophoresis of *16S rRNA* Replicon

The urinary *Enterobacter* spp. isolates, which were identified by morphological and biochemical assays, and were diagnosed by *l6S rRNA* gene PCR, showed a band length size of 465 bp (Figure 1).

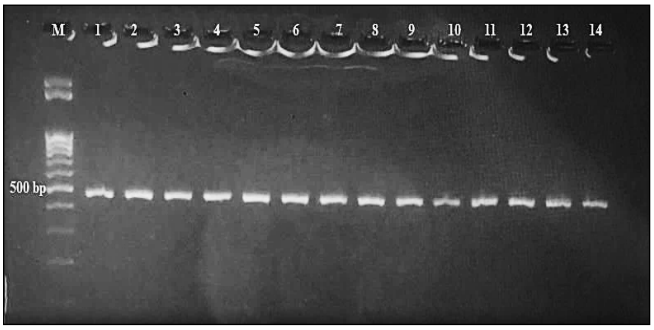

Figure 1. Gel Electrophoresis of PCR Products (465 bp) of l6S rRNA Visualised under UV Light after Staining with Ethidium Bromide

M: DNA Size Marker, 100–1500 bp Lanes 1–14: Number of Samples

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Sequencing Results of *Enterobacter* spp.

The *16S rRNA* sequences of *Enterobacter* spp. were amplified in part by using the position and size of the 465 bp PCR amplicons. Amplification of the NCB1 reference DNA sequence (Genbank accession number KM 979308.1) ranged from 292 to 756 base pairs. Regarding the alignment findings of the 465 bp, a comparison of the corresponding *16S rRNA* sequences revealed the existence of 13 mutations, 11 of which were found in S5–S7 and the other two in other bacterial isolates (Figure 2).

The exact sites of the identified mutations are documented in Table 1.

Figure 2.DNA Sequence Alignment of Seven Local *Enterobacter* spp. at 465 bp Amplicon with Their Analogous Reference 16S rRNA Sequences of *Enterobacter*

ref: NCBI reference sequences
s: Investigated isolates S1 to S7

Table 1.Pattern of the Observed Mutations (Transition A ↔ G or Transversion C ↔ T) in the 465 bp of *16S rRNA* in Appraisal with the NCBI Referring Sequences (Gene Bank Acc. No. KM9792008.1)

Isolate No.	Native	Mutation	Position in the PCR Fragment	Position in the Reference Genome (GenBank Acc. No. KM979208.1)
S1–S5	G	A	67	358
S2–S4	T	A	131	422
S5–S7	T	G	202	493

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S5–S7	A	G	212	503
S5–S7	A	T	213	504
S5–S7	C	T	223	514
S5–S7	A	G	238	529
S5–S7	C	T	249	540
S5–S7	C	T	253	544
S5–S7	C	G	290	581
S5–S7	A	G	297	588
S5–S7	G	T	341	632
S5–S7	G	T	364	655

S: Code of the isolate sample

Construction of *16S rRNA*–Based Phylogenetic Tree

Based on *16S rRNA* PCR amplicons, the current study generated a thorough phylogenetic tree. This phylogenetic tree included S1 through S7 samples in addition to other related DNA sequences. There were 115 aligned nucleic acid sequences from different *Enterobacter* species in this tree. A number of species were included, and other closely related creatures, like *Enterobacter cloacae*, *Enterobacter* ludwigii, *Enterobacter kobei,* and *Enterobacter asburiae,* were also mentioned. It is noteworthy that only two major phylogenetic clusters within this tree contained the examined local (Figure 3).

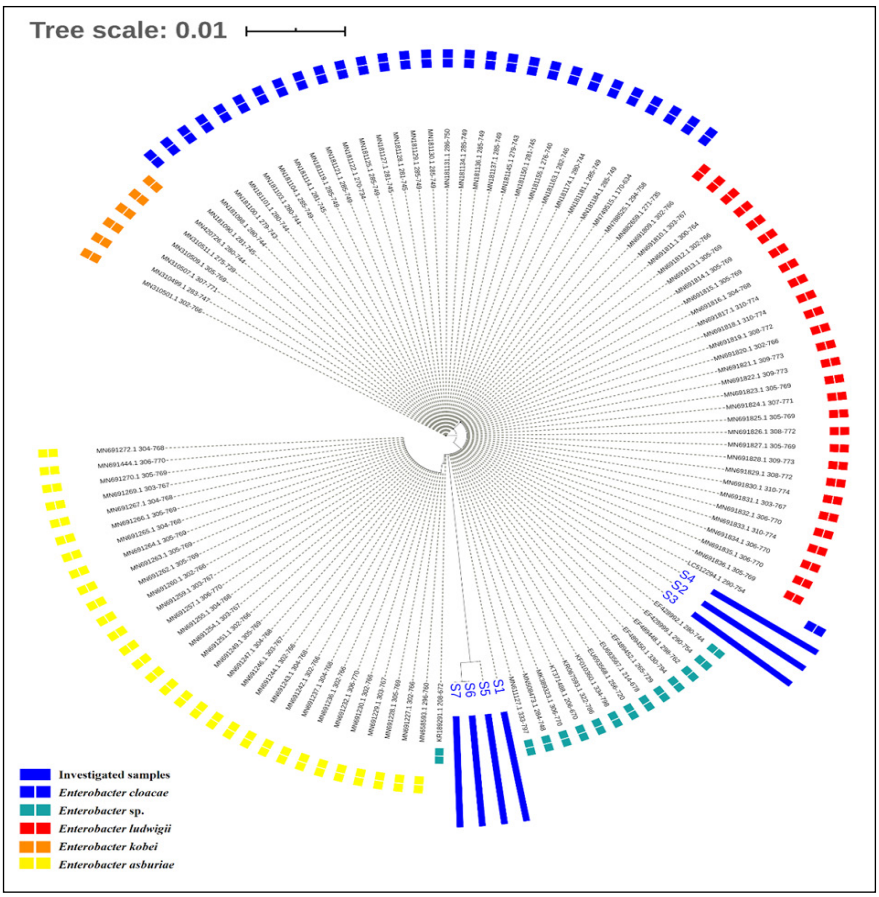

Figure 3.A Comprehensive Tree of Genetic Variants of the 16S rRNA Fragment of Seven *Enterobacter* spp. Isolates. The blue arrays colour speak of to the related referring NCBI deposited

Mentioned numbers: GenBank account number of each referring species

0.01 at the top portion of the tree: Degree of scale range among the comprehensive tree categorised organisms

S: Investigated isolates S1 to S7

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Discussion

The *Enterobacter* genus is generally found in the environment. *Enterobacter* infections can be acquired from either endogenous or exogenous sources. Among these bacteria, only a few species have been linked to nosocomial infections and epidemics. They are also implicated in community-acquired infections.[6] *E. cloacae* are the most common species found and described in clinical infections, mainly in immunocompromised patients in intensive care units, due to their resistance to antimicrobial agents and their opportunistic characteristics.[13,14]

The ability of *Enterobacter* spp. to invade and persist in the uroepithelium depends on several virulence factors and their ability to form biofilms.[6] Biofilm-forming bacteria are a common cause of recurrent and severe UTIs and are generally multidrug-resistant.[15]

There are a few studies concerned with the isolation rates of *Enterobacter* bacteria, as well as their resistance to antibiotics. One such study was a local study conducted by Al-Araji, who found that the prevalence rate of *Enterobacter cloacae* in UTI patients was 41.6%.[16] In two similar studies, the percentages of *Enterobacter* isolation were found to be 23.50% and 8.2%, respectively.[17,18]

The results of the molecular study showed that a special distribution of the isolates in the phylogenetic tree, in particular, S5–S7, have taken one unique clade within the tree in the immediate vicinity of two strains of *Enterobacter* spp. that isolated from Bangladesh and America, namely MN 611127.1 and KR18921.1, respectively. This is due to the common mutations exclusively detected in these samples. However, S1 was also incorporated in the vicinity of this clade because of one shared mutation G67A with S5, whereas S2–S4 had taken other positions that may be attributed to the presence of one mutation, T131 A, only in these samples. However, S2–S4 had taken more tilt toward the accession number LC 512294.1, which belongs to an Egyptian strain of *Enterobacter cloacae*. The genetic distance among all incorporated organisms within the *Enterobacter* sort had scored only 0.001, which manifested the very small genetic distances among these organisms.

Again, because of the currently detected mutations, the identities of S1 and S5–S7 scrutinised samples were determined as they belong to the same *Enterobacter* spp., while S2–S4 had taken a close phylogenetic position with *Enterobacter cloacae*. However, this notion provided a further identity of these studied local isolates.

Other *Enterobacter* spp. mentioned in this tree are *E. kobei, E. asburiae,* and *E. ludwigii*, which are important pathogens responsible for different contagions such as respiratory tract and intra-abdominal tract infections. *Enterobacter ludwigii* was isolated from the midstream urine of an 18-year-o1d man with nosocomial UTI.[19]

E. asburiae is a new strain under the *E. coloacae* complex first described as an unidentified strain under *Citrobacter* or *Enterobacter* and later named *E. asburiae*. It is an opportunistic pathogen isolated from different clinical specimens.[20]

Conclusion

Our phylogenetic analysis has observed the highest species detection specificity with regard to *16S rRNA*–based PCR. This phylogenetic protocol popularly gives a further indication of the power of the currently utilised *16S rRNA* specific primers to discriminate amongst the current strains. The presented tree has added an accurate and inclusive phylogenetic distribution and positioning of the seven studied isolates that originated due to the differences in nucleic acid substitutions within the same detected species.

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Declaration of Generative AI and AI-Assisted Technologies in the Writing Process: None

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