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Characterization and Molecular Identification of Unknown Bacteria Isolated From Outlet of Arab El Madabegh Sewage Treatment Plant in Assiut City, Egypt

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Abstract: The aim of this study isolation, characterization and molecular identification of unknown bacteria from the outlet of sewage treatment plant (Arab El Madabegh station) on the Nile River in the Assiut governorate, Egypt. Bacterial strain was isolated and characterized using various biochemical tests and confirmed through molecular approach. Bacterial 16S rRNA gene was amplified using suitable primers. The amplified 16S rRNA gene sequence was compared with the sequence in NCBI sequence database. The bacterial strain was identified as Salmonella .sp m33 (NCBI Gene Bank Accession No: KR027920.1) Phylogenetic and molecular evolutionary analyses were conducted using 16S rRNA sequencing. Salmonella .sp is a gram negative, rode shaped bacteria which forms white raised irregular colony. The sequence when submitted to NCBI gene bank database using BLAST showed 95 – 97% maximum identity and E-value equal to 0 for all closely related taxa.

Keywords: Salmonella, sewage, 16S rRNA, Isolation, characterization, NCBI.

1 Introduction

Large number of water borne microorganisms are pathogenic to humans, Cause many diseases. The transmission of these diseases through waste water is a constant public threat (4). Bacteria are the most important group of microorganisms found in this biological contact process, since they are responsible for the structural and functional activity of the activated sludge (11).

Biological treatment of wastewater is evaluated as a good treatment method for industrial and domestic effluents. Treatment of wastes with bacteria involves the stabilization of waste by decomposing them into harmless inorganic solids either by aerobic or anaerobic process. The processes used most frequently for biological treatment of wastewater in Common Effluent in Assiut, Egypt. Arab El Madabegh station one of sewage treatment plants in assiut governorate which discharge treated wastewater in Al Zennar drainage then in the Nile River which consider the main source of drinking water in Egypt.

Bacteria are generally identified by 16S rRNA sequencing. 16s ribosomal RNA is a component of the 30s Submit of prokaryotic ribosomes. One of the most attractive potential uses of 16s rRNA gene sequence informatics is to provide genus and species identification for isolates that do not fit any recognized biochemical profiles for strains generating only a low likelihood or acceptable identification according to commercial systems or for taxonomy that are rarely associated with human infectious diseases (5). The rRNA is the most conserved (least variable) gene in all cells. Portions of the rRNA sequence from distantly-related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria. Thus the comparison of 16S rRNA sequence can show evolutionary relatedness among microorganisms.

The 16SrRNA sequence has hypervariable regions, where sequences have diverged over evolutionary time. These are often flanked by strongly-conserved regions. Primers are designed to bind to conserved regions and amplify variable regions. The DNA sequence of the16S rRNA gene has been Determined for an extremely large number of Species. In fact, there is no other gene that has been as well characterized in as many species. Sequences from tens of thousands of clinical and

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environmental isolates are available over the internet through the NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov). These sites also provide search algorithms to compare new sequences to their database. Thus the present study is focused on the isolation, biochemical characterization and molecular identification of bacteria in treated sewage collected from the outlet of sewage treatment plant (Arab El Madabegh station) Assiut, Egypt and to characterize the isolates via biochemical tests and16s rRNA analysis.

2 Materials and Methods

The studies were carried out in Department of Microbiology, AL Azhar University Assiut branch , Assiut governorate, Egypt during 2015.

2.1 Isolation of Bacteria

The samples were collected from the outlet of sewage treatment plant (Arab El Madabegh station) Which flows into the drainage channel(Al Zennar drainage channel) and then on the Nile River in Assiut region , Egypt (Fig.1).

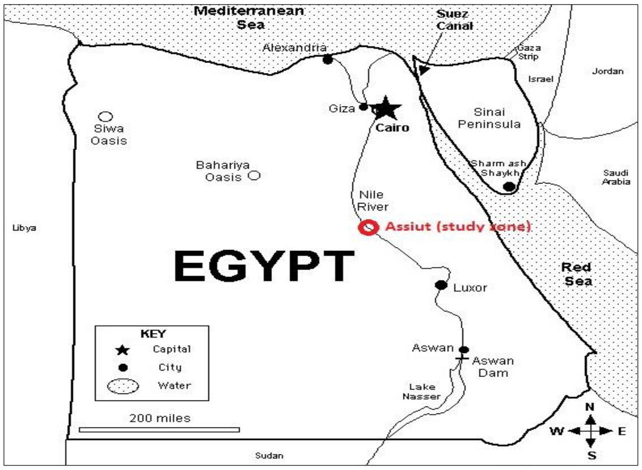

Figure 1: Map of sampling location (Al Zennar Drainag Discharge point in the Nile River Assiut, Egypt)

The samples were collected in a sterile plastic container and transported to laboratory for bacteriological analysis. Bacterial isolates were screened on Nutrient Agar (NA) plates by the standard pour plate method. Plates were incubated at 37°C/24h and a total of twenty isolates were obtained, from that one isolate was selected and used for further studies. The isolated bacteria were identified based on colony characteristics, gram staining methods and by various biochemical tests as given by Bergey's (3) Manual of Determinative Bacteriology.

2.2 Biochemical Characterization of the Bacteria

Selected strain was grown in nutrient broth culture medium containing 2.5% peptone, 1.0% yeast extract, and 0.5% beef extract. Cultures (50 ml in 250-mlconical flasks) were inoculated with 5% (v/v) inoculums and incubated at 37 °C with vigorous orbital shaking at 120-150 rpm. To make a solid medium, 1.5% agar was added to the broth. The shape and color of the colonies were examined under the microscope after Gram staining. Isolates were biochemically analyzed for the activities of Oxidase, Catalase, MR-VP test, Urease test, Motility, Indole production and Citrate utilization (Table. 1).

The tests were used to identify the isolates according to Bergey's (3) Manual of Determinative bacteriology.

Table 1: Biochemical characteristics of isolated strain

Test	**Result**
Gram stain	-
Motility	+
H2S	+
Urease	-
Citrate test	+
Indole test	-
Nitrate reduction	+
Oxidase	-
MR	+
VP	-
Lactose	-
Sucrose	-
Inositol	-
β-galaktosidase (ONPG Test)	-
Gas Production from glucose	+

2.3 Bacteria DNA Isolation and PCR Amplification of 16S rRNA Gene

Isolate was sent to SolGent Company Solgent Company, Daejeon South Korea for rRNA gene sequencing. Bacterial DNA was extracted and isolated using SolGent purification bead. Prior to sequencing, the ribosomal rRNA gene (also referred to as rDNA) was amplified using the polymerase chain reaction (PCR) technique in which Two universal primers were used for amplification: forward primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492r (5'-TACG GYTACCTTGTTACGACTT-3') were incorporated in the reaction mixture.

PCR products were purified and sequenced using a PCR purification kit (Cosmo Genetech, Republic of Korea) the purified PCR products were reconfirmed (using size marker) by electrophoreses on 1% agarose gel. Then these bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture. Each sample was sequenced in the sense and antisense directions using 27f and 1492r primers. Sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

Phylogenetic analysis is the study of evolutionary relationships among various groups of organisms. In accordance with the accumulated phylogenetic knowledge database, when comparing novel isolates with evolutionally closely related strains.

2.4 Computational Analysis (BLAST) and Identification of Bacterial Species

BLAST (Basic Local Alignment Search Tool) is a web based program that is able to align the search sequence to thousands of different sequences in a Database and show the list of top matches. This program can search through a database of thousands of entries in a minute. BLAST (1) performs its alignment by matching up each position of search sequence to each posit ion of the sequences in the database. For each position BLAST gives a positive score if the nucleotides match, it can also insert gaps when performing the alignment. Each gap inserted has a negative effect on the alignment score, but if enough nucleotides align as a result of the gap, this negative effect is overcome and the gap is accepted in the alignment. These scores are then used to calculate the alignment score, in “bits” which is converted to the statistical E-value. The lower the E-value, the more similar the sequence found in the database is to query sequence. The most similar sequence is the first result listed.

3 Results and Discussion

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level (8). The use of 16S rRNA gene sequences to study bacterial phylogeny (2) and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, J. B. 2001).The rRNA based analysis is a central method in microbiology used not only to explore microbial diversity but also to identify new strains. The present study has been carried out to identify the bacterial strain isolated from the outlet of sewage treatment plant (Arab El Madabegh station) Assiut, Egypt. Total of twenty isolates obtained, from that one isolate were used for further analysis. Isolated colony from mixed populations, on nutrient agar plates were characterized and sub cultured to obtain pure cultures, and the isolated bacteria were identified based on colony characteristics, and were biochemically analyzed for the activities of Oxidase, Catalase, MR-VP test, Urease test, Motility, Indole production, Citrate utilization (Table.1) and Gram stained (Fig.2) and was found to be Salmonella sp, further confirmation was done using molecular approach.

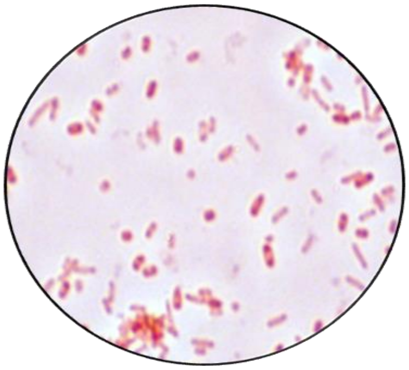

Figure 2: Microscopic View of selected isolate at 10 um

DNA was extracted and isolated using SolGent purification bead. Prior to sequencing, the ribosomal rRNA gene. The extracted DNA was used as template for amplification of 16S rRNA gene. The universal primers 27F and 1429R were used for the amplification and sequencing of the 16S rRNA gene fragment. The optimum annealing temperature was found to be 55ºC. An intense single band was visible on 1% agarose gel stained with ethidium bromide (Fig.3).

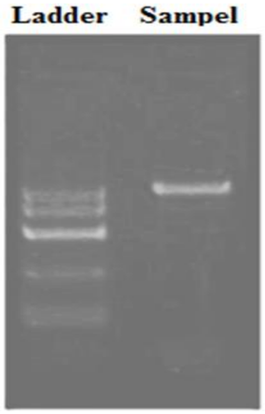

Figure 3: Sample 1200bp PCR product DNA Ladder 100bp, 250bp, 750bp and 1000bp.

The sequences (Fig.4) obtained were compared with the NCBI gene bank database using BLAST search program (http;//www.ncbi.nlm.nih.gov) (6), (8). The percentages of sequence matching were also analyzed. The homology search made using BLAST showed 96 % maximum identity with that of Salmonella enterica subsp. enterica serovar Typhimurium strain ATCC 13311 16S ribosomal RNA gene, NCBI Gene Bank Accession No: NR_119108and E-value equal to 0 for all closely related taxa. Further the sequences of the bacterial isolates were used for the construction of the phylogenetic dendrogram to know the genetic relatedness between the bacterial isolates. All the closely related homologs of identified bacteria were used for the construction of the phylogenetic dendrogram (Fig.5) to know their evolutionary origin. The dendrogram showing the relation between as Salmonella .sp m33 and their close homologs is shown in (Fig. 5*). Salmonella are zoonotic pathogens, as they can cause food-borne illness in humans (10).*

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   1 aag atg tgc ctt gaa gag ttt gat cat ggc tca gat tga acg ctg gcg gca ggc cta aca cat gca agt cga acg
  76 gta aca gga agc agc ttg cnc ttc gct nnn nac ggg tga gta atg tct ggg aaa ctg cct gat gga ggg gga taa
 151 cta ctg gaa acg gtg gct aat acc gca taa cgt cgc aag acc aaa gag ggg gac ctt cgg gcc tct tgc cat cac
 226 aga tgg gat tag cta gtt ggt gag gta acg gct cac caa ggc gac gag tgg cgg atc cct agc tgg tct gag agg
 301 atg acc agc cac act gga act gag aca cgg tcc aga ctc cta cgg gag gca gca gtg ggg aat att gca caa tgg
 376 gcg caa gcc tga tgc agc cat gcc gcg tgt atg aag aag gcc ttc ggg ttg taa agt act ttc agc ggg gag gaa
 451 ggt gtt gtg gtt aat aac aat tga cgt tac ccg cag aag aag cac cgg cta act ccg tgc cag cag cgg taa tac
 526 gga ggg tgc aag cgt taa tcg gaa tta ctg ggc gta aag cgc acg cag gcg gtc tgt caa gtc gga tgt gaa atc
 601 ccc ggg ctc aac ctg gga act gca ttc gaa act ggc agg ctt gag tct tgt aga ggg ggg tag aat tcc agg tgt
 676 agc ggt gaa atg cgg cct aga gat ctg gag gaa tac cgg tgg cga agg cgg ccc cct gga caa aga ctg acg ctc
 751 agg tgc gaa agc gtg ggg agc aaa cag gat tag ata ccc gca gcc tgg tag tcc acg ccg taa acg atg tct act
 826 tgg agg ttg tgc cct tga ggc gtg gct tcc gga cgt aac gcg tta agt aga ccg cct ggg gag tac ggc cgc aag
 901 gtt aaa act caa atg aat tga cgg ggg ccg cac aag cgg tgg agc atg tgg ttt aat tcg atg caa cgc gaa gaa
 976 cct tac ctg gtc ttg aca tcc aca gaa gaa tcc aga gat gga ttg gtg cct tcg gga act gtg aga cag gtg ctg
1051 cat ggc tgt cgt cag ctc gtg ttg tga aat
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Figure 4: Partial sequence of PCR product of 16S rRNA gene sequence of isolated Bacteria.

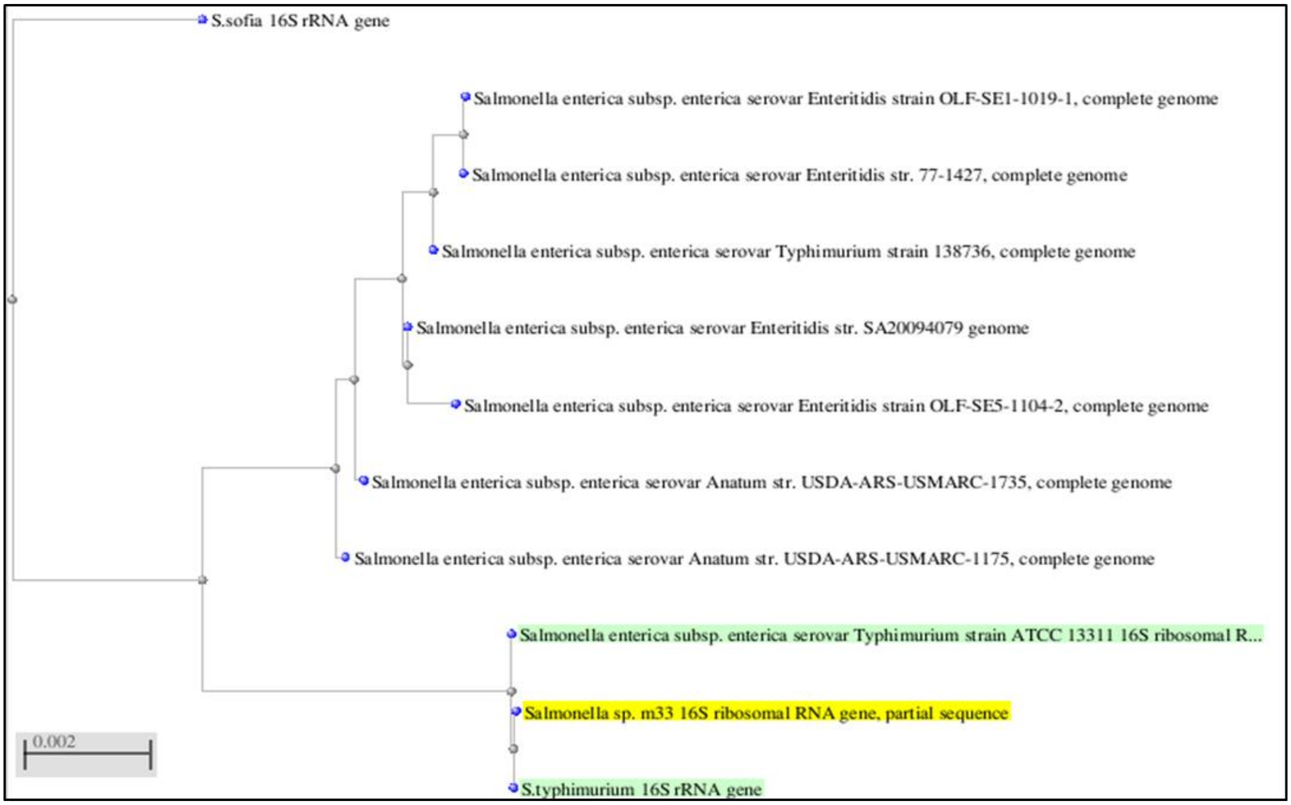

Figure 5: Phylogenetic tree representing close homologs of Salmonella sp. M33 strain.

4 Conclusion

16s rRNA gene sequencing provide genus and species identification for isolates that do not fit any recognized biochemical profiles for strains generating only a low likelihood or acceptable identification according to commercial systems or for taxa that are rarely associated with human infectious diseases. the use of 16S rRNA gene sequences to identify new strains bacteria is gaining momentum in recent years. We showed the use of 16S rRNA gene sequence to characterize the bacterial isolate from the outlet of sewage treatment plant (Arab El Madabegh station) Assiut, Egypt. and were found to be Salmonella .sp m33 (NCBI Gene Bank Accession No: KR027920.1) . Thus, the genotyping method using 16S rRNA gene sequence is both simple and effective in strain identification.

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