Enterobacter cloacae identification by 16S rRNA and rpo B gene sequencing of the Cichlidae Tilapia fish pathogen in Babel local market

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Abstract

This study was carried to investigate the presence of Enterobacter cloacae pathogen in fish Tilapia (n = 21), Liza abu (n = 9). A total of 30 samples from different region of fish, routine bacteriological examination were done and evaluation of isolates by PCR assay using of 16S rRNA and rpo B genes, The results was showed that 11 Enterobacter cloacae isolates tested and produced bands with the same intensity as the standard PCR performed using pure DNA. The genetic methods allowed detecting the virulence factors of bacteria. According to 16S rRNA sequence comparisons, the type strain of E. cloacae showed closely related to Enterobacter species similarity levels (97.8%) when compared with references strains in Genbank. Phylogenetic tree was distributed by neighbor- goining method appeared closely related to E. cloacae. The results of the present study constitute an advance in the available diagnostic methods for bacterial pathogens in fish markets.

Keywords: *Fish, Enterobacter cloacae, DNA, 16S rRNA fish Tilapia.*

INTRODUCTION

To avoid and control diseases caused by Enterobacter cloacae, it is crucial to evaluate the following factors: epizootiology, antigenicity, biochemistry, pathogenesis, etiological agents, zoonosis disorders, and inter-relationship environmental factors and stress. However, farming of fish is a relatively new job; these aspects have not been thoroughly investigated (1). Recently, although paying more attention to the detection of agents of bacterial etiological there are still limited options for controlling fish bacterial infections in Babel. As a result, faster and more effective diagnostic options are required. This would aid in disease control before permanent clinical implications and high mortality rates occur. Molecular diagnostic methods detect pathogens with high sensitivity, specificity, and accuracy using little amounts of sample material. (2). Since of no need to extract pure DNA, methods (in addition to PCR) are faster and less expensive, and they can help discover fish diseases early. (3). It is good to utilize degenerate and universal since not all

sequences of microorganism are cataloged in databases. As a result, the approaches based on sequencing and amplification of the 16S rRNA ribosomal gene have received much attention. (2). In the environment, the concept behind the usage of universal PCR primers is that the primers are complementary to genes' conserved portions (leading to amplification, as a consequence). Then, in hypervariable regions, heterogeneity is then observed inside the fragments flanked by the primers. (4) (5). From investigations of non-cultivable bacteria to identifying and accurately diagnosing diseases, this technology has revolutionized microbial ecology. To identify fish Enterobacter cloacae pathogens, this study aimed to evaluates 16S rRNA paired with rpo B gene sequencing to find whether it is less expensive and faster in comparison with the traditional PCR method. Furthermore, for the practical tests of aquaculture illness diagnostics, utilizing the methods mentioned above demonstrates the efficacy of genetic methodologies in Babel farms.

Materials and methods

Ethically Approved This study was approved by the ethical and research committee of 1Department of Microbiology, College of Veterinary Medicine, Al-Qasim green university, Iraq. With approving in University of Baghdad, Dept. of Internal and Preventive Vet. Medicine.

Bacterial Strains and Culture Conditions:

Thirty samples were obtained from fish of two species: Tilapia (n = 21), Liza abu (n = 9). The fish seemed with healthy appearance, samples were collected from different regions of fishes: skin scraping, blood and brain.

For the isolation of Enterobacter cloacae bacteria, scrapings were performed using sterile swabs from skin and brain and blood was collected. These samples were cultured on on different selective media, XLD (Xylose Lysine Deoxycholate agar), and SS (Salmonella Shigella), and incubated for 24 hrs in bacteriological incubator adjusted to 37˚C. Gram stain and biochemical were done on isolated bacteria (Quinn et al 2004). Confirmation of isolates in the National Center of health laboratories / Enterobacteriacae unit /Ministry of Public Health / / Baghdad.

Molecular Identification

DNA extraction: Fresh bacterial genomic DNA was extracted using (A Bosphore® Bacterial Manual DNA Extraction Spin Kit Anatolia Gene Works, Turkey) from 1ml nutrient broth samples in 1.5ml microcentrifuge tubes. At 260 nm absorbance and kept at 20 °C until required, the concentration of pure genomic DNA was determined via a NanoDrop-1000 spectrophotometer (NanoDrop Technology, USA).

Primers: two primers were used in this study (Table, 1)

Table (1): The primer sequences for 16S rRNA and rpoB

Primer	Sequence (5'-3')		product size (bp)	Source
rpoB	R	CCT GAA CAA CAC GCT CGG A	1090bp	Mollet *etal*.,1997
	F	AAC CAG TTC CGC GTT GGC CTG G		

16S rRNA	F	CCAGGTAAGGTTCTTCGCGT	770bp	Suarez *et al*., 1997 and Lomnitz *et al*.,2016
	R	GGAACTGAGACACGGTCCAG		

PCR amplification

As illustrated in Table 1, the published PCR primer sets are used to develop two PCR protocols in order to amplify 16S rRNA and rpoB of En regions. 50 µL PCR reaction contained 25 µL of HotStarTaq Master Mix (Kappabiosystem, USA) for 16S rRNA PCR amplification .With the last concentration of 1.5 mM MgCl2 and 200 µM each dNTP, the premixed solution consists of dNTPs, PCR Buffer and HotStarTaq DNA Polymerase. Template DNA (50 ng) and 1.5 mM of additional MgCl2 (Kappabiosystem, USA) were diluted in PCR-grade water. 25 µL of a solution consisting of 200 nM of each primer. In a SureCycler PCR8800 thermocycler (Agilent Stratagene, Santa Clara, CA, USA), for 35 cycles, the PCR reactions were run. Each cycle is 72 °C for 60 s, 60 °C for 45 s and 94 °C for 45 s). The initial hot started (94 °C for 15 m) and a final extension (72 °C for 10 m). Except for the 50 °C for the O-antigen PCR amplification and the annealing temperature was 56 °C for rpoB, the PCR conditions for rpoB were similar to 16S rRNA. The agarose gel electrophoresis evaluated the PCR products and then visualized them after the ethidium bromide stain.

Gene Sequencing and Purification of PCR Products

As confirmed by PCR, all positive isolates have been sequenced to confirm the bacterial genome type. PCR products, including 16S rRNA and rpo B genes, were purified using the PCR Clean UP-kit (Promega, Co., USA) based on instructions of the manufacturer for sequencing analysis. At the Seoul national university (South Korea)/college of agriculture and life sciences/ national instrumentation center for environmental management, sequencing has been performed. According to the manufacturer's recommendations, both forward and reverse primers have been used to perform the sequencing reactions in 10 µl reaction volume (abi big dye v3.1 ready-reaction kit; applied biosystems, USA). 3730xl DNA analyzer (applied biosystems, USA) has been utilized to analyze the samples. Ape (a plasmid editor) software (v2.0.49, 2015) was used to align forward and reverse complementary sequences. The determined outcomes have been sent to Genbank. On the Genbank database, the analysis was performed via blast search (http://blast.ncbi.nlm.nih.gov/). The program is online and developed by National Center for Biotechnology Information (NCBI). Usually, it is used to identify sequences of the hits of the subject that are stored in international nucleotide databases (such as DDJD, EMBL, GenBank, etc.). It may give good matching with the query sequence. Multiple sequence alignment (MSA) for the query-sequence and similar subject sequences were carried out using Mega X software. Based on the multiple alignment result, a phylogenetic tree was constructed via Mega X software to identify the taxonomic affiliation of each unknown bacterial isolate with closely related bacteria.

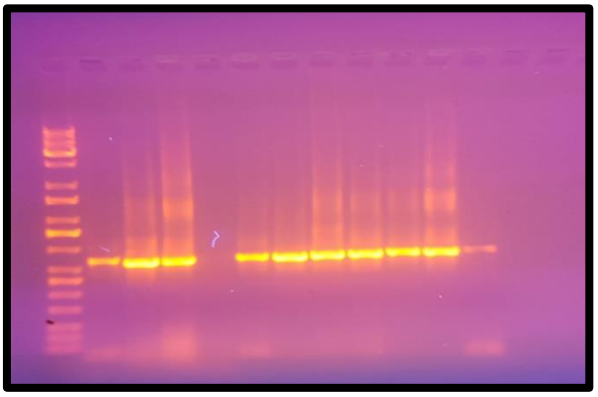
Results and Discussion

Enterobacter isolation and Identification

from 30 fishes, samples collected from various fishes organs. The results revealed 11 isolates of Enterobacter cloacae grew in XLD and SS agar were confirmed in the central health laboratories / Enterobacteriace unit/ Bagdad. This 11 isolated from skin mucus (n =6), blood (n =3), brain (n = 2).

Molecular identification:- Molecular technique based on 16S rRNA sequencing is utilized to determine the quest bacterial strain at the species level. As illustrated in Figure 1, the partial sequence (770 bp) of the 16S rRNA gene was successfully amplified.

Figure (1): Agarose gel electrophoresis image that shows the PCR product of Enterobacter cloacae partial 16S rRNA gene

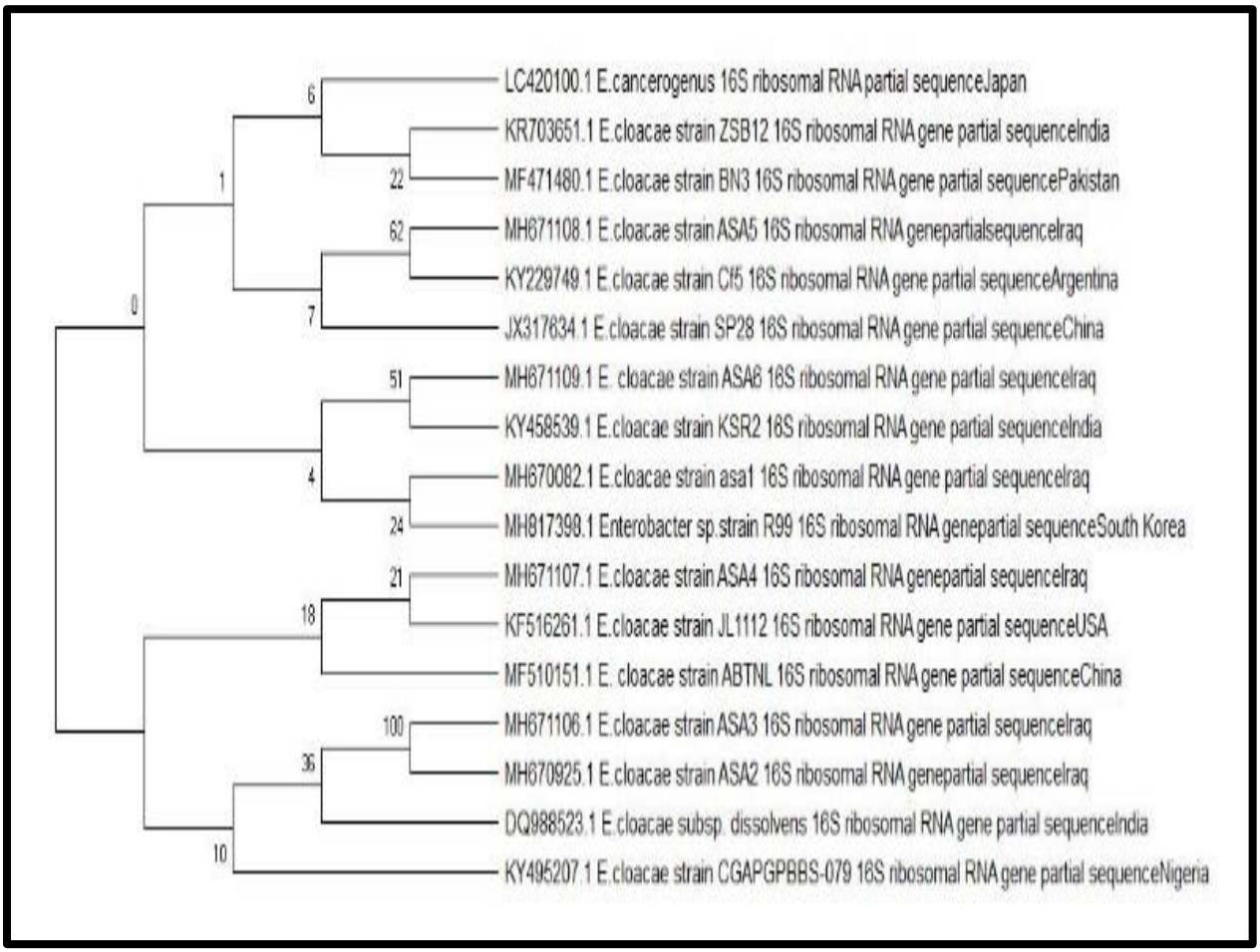


The phylogenetic relationship among 16S rRNA sequences of bacterial isolates belonging to Enterobacter cloacae

Of the 16S rRNA gene, partial nucleotide sequences from six bacterial isolates of the quest have been determined after DNA sequencing. The lengths are as follows: ASA6 (453 bases), ASA1(614b), ASA4(621b), ASA3(620b), ASA2(620b), ASA5(631). As shown in Figure 2, the result in MSA and phylogenetic trees (Figure2) conferred that16S rRNA nucleotide sequences stored in the databases of international nucleotide with accession numbers and similarity percentage as follow:

ASA6(gb:MH671109.1),ASA1(gb:MH670082.1),ASA4(gb:MH671107.1),ASA3(gb:MH671106.1),ASA2(gb:MH670925.1),ASA5(gb:MH671108.1).

Figure (2) Neighbor-joining tree shows the phylogenetic relationship among 16S rRNA sequences of bacterial isolates (isolated from local fish in Iraq) belonging to Enterobacter cloacae and other 16S rDNA sequences belonging to closely related bacteria. Their accession numbers in international nucleotides are used to express the hits.



Molecular identification and phylogenetic relationship among rpoB gene sequences of Enterobacter cloacae

The confirmation process of the E. cloaca isolates recovered from fish samples to detect the presence of rpoB gene by PCR, All 11 isolates were possess this gene equal to target product size(1090bp) (Figure 3).

The phylogenetic relationship among rpo B sequences of 6 bacterial isolates (isolated from local fish in Iraq) belonging to Enterobacter cloacae and other rpo B sequences(Figure 4).

Figure (3): Agarose gel electrophoresis image that shows the PCR product of Enterobacter cloacae partial rpoB gene.

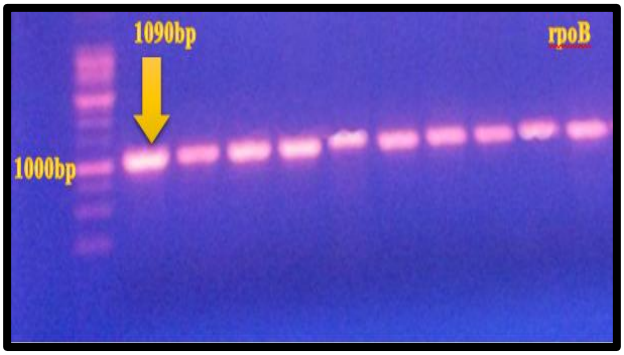
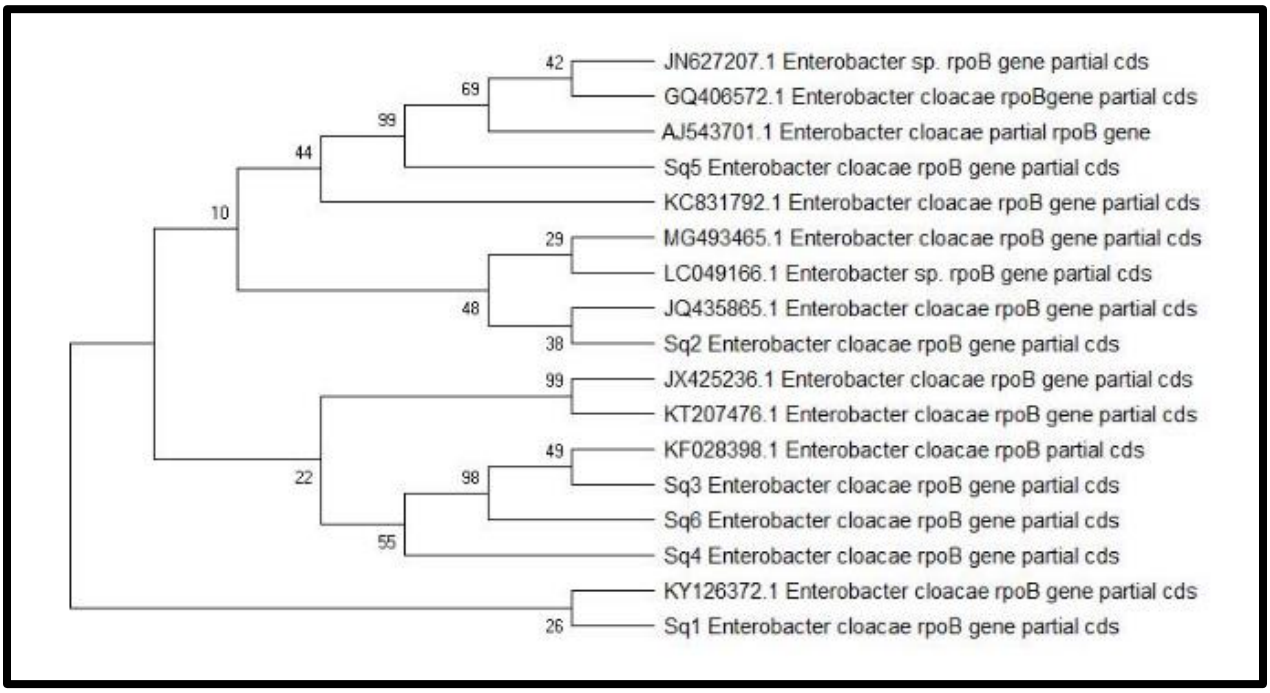


Figure (4) Neighbor-joining tree showing the phylogenetic relationship among rpo B sequences of 6 bacterial isolates (isolated from local fish in Iraq) belonging to Enterobacter cloacae and other rpo B sequences belonging to closely related bacteria. Hits are expressed by their accession numbers in international nucleotide.



Within a species, the following terms can be used to express the microheterogeneity concept: variant subspecies genotypes, intraspecies variation, and Sequevars. It often defines the differences of less than 0.5% or only a few base pairs per 16S rRNA gene sequence. No total agreement is performed to put the name of organism groups that show microheterogeneity. Microheterogeneity is essential to clinical microbiologists because it allows the ability to identify important pathogenicity, phenotype, and niche differences between strains (6,7). Additionally, microheterogeneity was exploited for strain tracking and epidemiological research.

(8) Comparing partial sequences of 78 enterobacterial species in the phylogeny of clinical samples was analyzed by their F-ATPase b-subunit gene and their elongation factor Tu gene. Based on the 16S rRNA gene sequences, analogous trees (exist in the databases) have been reconstructed

As seen in Figure 1, the sequencing data is validated by building A maximum-likelihood phylogenetic tree. As anticipated, into a common branch, the bacterial isolates of phylogenetic related or the same species have been accurately grouped. For phylogenetic inference, the maximum likelihood principle examines the given model probability of evolutionary changes describing data observed origin. In the method, the neighbor-joining method is used to construct the initial tree. To maximize the likelihood and for the desired evolutionary model, each branch's length is changed so that the data will introduce tree topology (9).

According to the sequence of 16S rDNA comparisons, the kind strain of E. cloacae was more closely related to related bacteria from

Enterobacter species (97.8% similar). The polyphyletic character of Enterobacter spp. is supported by our findings. Sequence comparisons of the 16S rDNA and the rpo B (10). Phred quality scores of 20 were found in electropherograms obtained from rpo B gene sequencing. The bacterial identification results for both genes were the same in all isolates. Graph (2). To compare with the published sequences for Borrelia burgdorferi and Treponema pallidum, the gene encoding the RNA polymerase beta subunit (rpoB) targeting for the Leptospira biflexa (serovar patoc) . (11)

(12) have been evaluated with a combination of sequence and PCR-restriction fragment length polymorphism (PCR-RFLP) analyses of the three housekeeping genes hemB, rpoB, and hsp60 (in addition to ampC), the gene of a class C β-lactamase. In addition to all 13 type strains of the genus, two hundred and six strains were gathered from twenty-two agricultural centers, veterinarians, and hospitals in eleven countries (13). It represents a significant variable side of the gene in the case of comparing salmonella enteric Typhimurium and complete rpo B sequences of E. coli

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