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Phylogenetic Analysis of *Bacteroides fragilis* based on Neuraminidase gene Isolated from Chronic Tonsillitis.

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ABSTRACT

Chronic tonsillitis represents the most common inflammatory lesions of the pharynx occurring predominantly in the younger age group; it is caused by pathogenic bacteria. *Bacteroides fragilis* is opportunistic pathogen, and the predominant obligately anaerobic organism isolated from abscesses and soft tissue infections, as well as diarrheal diseases in animals and humans. In this study the phylogenetic relationships between eight local isolate of *Bacteroides fragilis* and *Bacteroides fragilis* global isolates were analyzed using the neuraminidase gene sequence. The DNA sequencing analysis was performed by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree Test) by MEGA 6 program based analysis of 582bp neuraminidase gene (*nanH*) was amplified by polymerase chain reaction. Phylogenetic analysis results of these gene sequences revealed that *B. fragilis* local number (S1, S2, S3, and S7) isolates were closely related to isolation *B. fragilis* recorded globally NCBI-BLAST *B. fragilis* (M31663.1), and results of the analysis showed a clear convergence of isolates of *B.fragilis* local number (S5 and S6) to the isolation of *B.fragilis* recorded globally NCBI-BLAST *B.fragilis* (D28493.1) and (Af031639.1) then this study recorded two unique local isolation of *B.fragilis* number (S4 and S8) differ from isolation recorded globally

Keywords: *Bacteroides fragilis* , Phylogenetic analysis, neuraminidase .

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INTRODUCTION

Chronic tonsillitis is the most common disease in the throat occurring predominantly in the younger age group. It is a condition caused by recurrent infections of tonsils caused by aerobic and anaerobic pathogenic bacteria (23,10). *Bacteroides fragilis* the predominant obligately anaerobic organism isolated from abscesses and soft tissue infections, as well as diarrheal diseases in animals and humans (8,15,21,16). It is a pleomorphic, encapsulated, non-motile, nonspore-forming and a gram-negative rod. It varies in size from 1.5 to 6 µm long. It does not ferment arabinose, melezitose, or salicin, but grows well in the presence of 20%bile, is indole-negative and catalase-positive, Constitutes 1% to 2% of the normal colonic bacterial micro flora in humans (11).

The high frequency of *B. fragilis* involvement at reflects the actions of several virulence factors. The enzyme neuraminidase (NANase) production is mediated by the *nanH* gene, which is present in several pathogens including *Streptococcus pneumonia* (3), *Salmonella typhimurium* (19, 13), *Vibrio cholerae* (13), *Corynebacterium diphtheriae* (14), and *B. fragilis* (7). It has been suggested that NANase plays a role in the pathogenicity of these bacteria as well as in infections caused by the protozoan *Trypanosoma cruzi* (5) and the influenza virus (1).

The neuraminidases, or sialidases, are a group of glycohydrolase enzymes that cleave N-acetyl neuraminic acid (NANA [sialic acid]) residues from the termini of simple and complex oligosaccharides, glycolipids, gangliosides, and glycoproteins. Sialic acids are widely distributed on the surfaces of all eukaryotic cells as terminal no reducing residues and on important glycoproteins, such as immunoglobulin G and the Clq component of complement (4, 22). In the latter, the presence or absence of sialic acids from the surfaces of cells and from these important glycoproteins may serve the nutritional requirements of the bacteria as well as disrupt many host functions (2,6,18,12,20). In this study the neuraminidase gene was examined and analyzed for construction of phylogenetic trees of local Iraqi *Bacteroides fragilis* in comparison to other *Bacteroides fragilis* globally registered.

MATERIALS AND METHODS

Samples collections

This study includes analysis of 112 patients who underwent tonsillectomy at Al-Diwaniyah Teaching Hospital for the period from October 2014 to May 2015. The tonsils were divided into 2 groups, first group include 112 tonsil and it was obtained 30 isolation of *B.fragilis* diagnosed by bacteriological standard methods and Polymerase chain reaction technique (PCR). The other group 112 has been getting it on 57 isolated from *B. fragilis* diagnosed by direct polymerase chain reaction technique (Direct PCR) and both molecular methods using gene (*nanH*).

Bacterial and Tissue Genomic DNA Extraction

Genomic DNA was extracted from bacterial and tonsil tissue by using (Genomic DNA Mini Kit, Geneaid. USA). The extraction was done according to company instructions. After that, the extracted gDNA was checked by Nanodrop spectrophotometer, and then stored at -20C at refrigerator until used in PCR amplification.

Polymerase Chain Reaction (PCR)

PCR assay was carried out by using specific primer which was designed by using neuraminidase gene genetic sequences found in GenBank at the National Center for Biotechnology Information (NCBI) and using design prefixes Primer3 plus program, to amplify a 528bp fragment of highly conserved regions of neuraminidase gene in *B.fragilis*. Neuraminidase forward primer (CCGGTAGTTCAAGCAGCAGA) and neuraminidase Reverse primer (GCGCACTCCTACTCCCATAC) were provided by (Bioneer Company. Korea). Then PCR master mix was prepared by using (AccuPower® PCR PreMix kit. Bioneer. Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl2 1.5mM, stabilizer, and tracking dye) and the PCR master mix reaction was prepared according to kit instructions in 20µl total volume by added 5µl of purified genomic DNA and 1.5µl of 10pmole of forward

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primer and 1.5µl of 10pmole of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20µl and briefly mixed by Exispin vortex centrifuge (Bioneer. Korea). The reaction was performed in a thermocycler (Techne TC-3000. USA) by set up the following thermocycler conditions; initial denaturation temperature of 95 °C for 5 min; followed by 30 cycles at denaturation 95 °C for 30 s, annealing 55 °C for 30 s, and extension 72 °C for 30 s and then final extension at 72 °C for 7 min. The PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

DNA sequencing method

DNA sequencing method was performed for detection Phylogenetic relationship of *B.fragilis* based on neuraminidase gene by Phylogenetic tree analysis. 528bp PCR product was purified from agarose gel by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada). The purified neuraminidase gene PCR product samples were sent to Macrogen Company in South Korea for performed the DNA sequencing by (AB DNA sequencing system).

RESULTS

Out of 224 samples of tonsils of 112 patients undergoing tonsillectomy were tested by convential PCR assay, only 87 samples which appeared positive for *B.fragilis* at 528bp PCR product of neuraminidase gene on agarose gel electrophosis (Fiq.1).

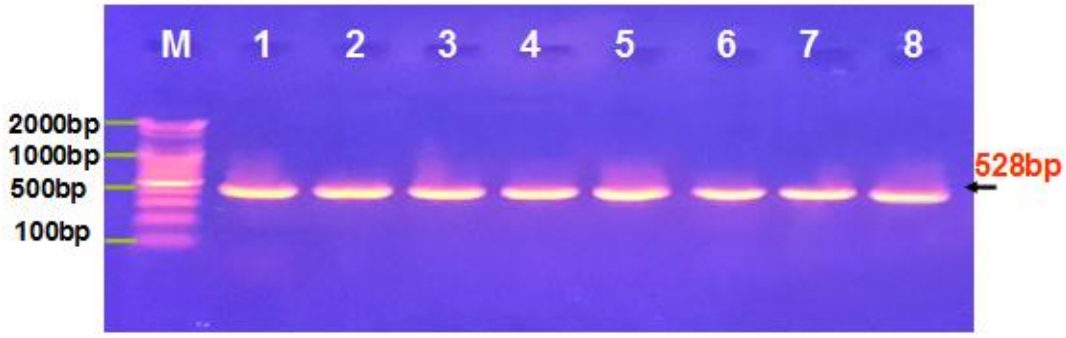

Figure (1): Agarose gel electrophosis image, which show the PCR product results for *B.fragilis* of Neuraminidase gene at 528bp PCR product size, where M: Marker 100bp, Lane (1-8) are positive samples.

The DNA sequencing analysis to product of eight samples positive for *B.fragilis* was performed to confirm the PCR results, by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree Test) by MEGA 6 program, showed phylogenetic sequence of (*B.fragilis* local isolate) number (S1, S2, S3, and S7) was more closely related to isolation *B. fragilis* recorded globally NCBI-BLAST *B. fragilis* (M31663.1). And results of the analysis showed a clear convergence of isolates of *B.fragilis* local number (S5 and S6) to the isolation of *B.fragilis* recorded globally NCBI-BLAST *B.fragilis* (D28493.1) and (Af031639.1).While isolated *B. fragilis* local number (S4 and S8) appeared different from the rest of the other apparent in the analysis of the genetic tree (Fig.2).

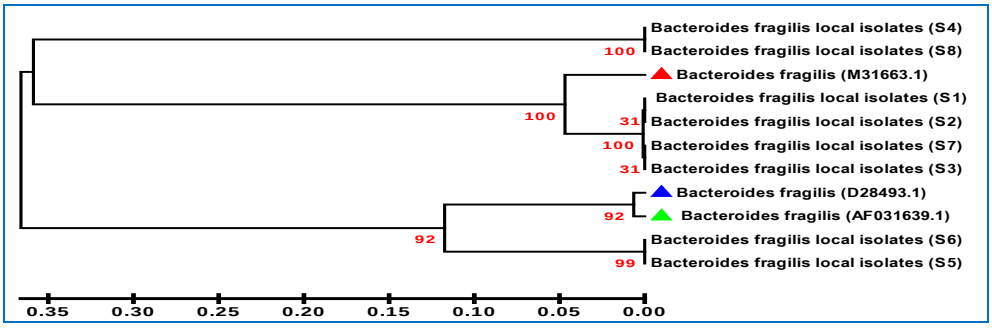

Fig.2: The phylogenetic tree of selected eight B.fragilis based upon neuraminidase gene using Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree Test) by MEGA 6 program.

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The DNA sequencing analysis of neuraminidase gene, 528bp PCR product of *B.fragilis* showed in (Fig.3).

>B.fragilis nanH gene_S1

GTATGGGAGTAGGAGTGCGCCATGCCGGAGACGATAATTCTGCCGCATTCCGTATTCCGGGATTGGTGAC
TACTAATAAAGGTACGTTACTGGGAGTTTATGATGTACGTTATAACAGTAGTGTTGACTTGCAGGAGCAT
GTTGATGTTGGTTTAAGCCGCAGTACAGATGGTGGAAAAACTTGGGAAAAAATGCGTTTGCCTTTGGCTT
TTGGAGAATTCGGTGGTTTGCCTGCCGGTCAGAATGGGGTAGGAGACCCTTCTATTCTTGTTGATACAAA
AACAAATAATGTTTGGGTGGTTGCTGCCTGGACACATGGTATGGGTAATCAGCGGGCATGGTGGAGTTCA
CATCCGGGTATGGATATGAACCATACAGCACAACTTGTTTTAGCTAAAAGTACAGATGATGGTAAAACAT
GGTCTGCACCTATTAATATTACAGAGCAGGTGAAAGATCCTTCTTGGTATTTCTTGTTGCAGGGACCGGG
TAGGGGTATCACT

>B.fragilis nanH gene_S2

AAGCCGCAGTACAGATGGTGGAAAAACTTGGGAAAAAATGCGTTTGCCTTTGGCTTTTGGAGAATTCGGT
GGTTTGCCTGCCGGTCAGAATGGGGTAGGAGACCCTTCTATTCTTGTTGATACAAAAACAAATAATGTTT
GGGTGGTTGCTGCCTGGACACATGGTATGGGTAATCAGCGGGCATGGTGGAGTTCACATCCGGGTATGGA
TATGAACCATACAGCACAACTTGTTTTAGCTAAAAGTACAGATGATGGTAAAACATGGTCTGCACCTATT
AATATTACAGAGCAGGTGAAAGATCCTTCTTGGTATTTCTTGTTGCAGGGACCGGGTAGGGGTATCACTA
TGAGTGACGGTACTTTGGTATTCCCAACTCAGTTTATCGATTCGACACGTGTGCCCAATGCCGGTATTAT
GTACAGTAAAGATGGTGGCAAGAACTGGAAGATGCACAATTATGCACGTACGAACACCACAGAAGCCCAG
GTAGCTGAGGTCGAACCCGGAGTGTTGAT

>B.fragilis nanH gene_S3

AAGCCGCAGTACAGATGGTGGAAAAACTTGGGAAAAAATGCGTTTGCCTTTGGCTTTTGGAGAATTCGGTG
GTTTGCCTGCCGGTCAGAATGGGGTAGGAGACCCTTCTATTCTTGTTGATACAAAAACAAATAATGTTTGG
GTGGTTGCTGCCTGGACACATGGTATGGGTAATCAGCGGGCATGGTGGAGTTCACATCCGGGTATGGATAT
GAACCATACAGCACAACTTGTTTTAGCTAAAAGTACAGATGATGGTAAAACATGGTCTGCACCTATTAATA
TTACAGAGCAGGTGAAAGATCCTTCTTGGTATTTCTTGTTGCAGGGACCGGGTAGGGGTATCACTATGAGT
GACGGTACTTTGGTATTCCCAACTCAGTTTATCGATTCGACACGTGTGCCCAATGCCGGTATTATGTACAG
TAAAGATGGTGGCAAGAACTGGAAGATGCACAATTATGCACGTACGAACACCACAGAAGCCCAGGTAGCTG
AGGTCGAACCCGGAGTGTTGAT

>B.fragilis nanH gene_S4

ATAATCGTGGAGGAAGCCGCGCTGTGGCTATTACAAAAGATTTGGGTAAAACATGGACGGAACATGAATC
TTCTCGTAAGGCATTGCCGGAATCTGTTTGTATGGCTAGTTTGATCAGTGTGAAAGCAAAAGATAATGTG
TTGGGCAAGGATTTATTGATTTTCTCTAATCCTAATACGACAAAAGGACGCTATAATACTACTATTAAAA
TAAGTTTGGATGGCGGTGTGACTTGGTCACCCGAACATCAGCTGTTGTTGGATGAAGGCAATAACTGGGG
TTATTCTTGCCTCTCTATGATTGATAAAGAGACGATTGGTATTCTTTATGAAAGTAGTGTGGCGCACATG
ACATTCCAGGCTGTGAAGTTGAAAGACATTATTAAATGAGAGATTTCCCCCATAAACTAAATAAATACCG
ATGAGAAACAAACAAATTTTCTTAATCAGTTTCATTATTTACTTATGCACAATTTCTTCGGTTCGGGCAG
GTGAATATCACTTCCCGGAACCGAAAA

>B.fragilis nanH gene_S5

CCGGTAGTTCAAGCAGCAGATACCATTTTTGTTCGTGAAACACGTATTCCTATTTTGATAGAAAGGCAGG
ATAATGTCTTATTCTATCTCCGTTTGGATGCTAAAGAGAGCCAGACCCTGAACGATGTAGTGTTAAACTT
GGGTGAAGGAGTGAATCTTTCTGAAATTCAGTCCATAAAGCTTTATTATGGAGGTACTGAAGCATTGCAG
GATAGTGGTAAAAAACGTTTTGCTCCTGTTGGATATATTTCCAGTAACACTCCGGGGAAAACACTTGCCG
CCAATCCGTCTTATTCAATCAAAAAATCAGAGGTGACTAATCCCGGTAATCAGGTTGTTCTGAAAGGAGA
TCAGAAACTGTTTCCCGGAATCAATTATTTCTGGATTAGTTTGCAAATGAAGCCGGGCACTTCGCTTACC
AGCAAGGTGACCGCGGATATCGCTTCAATTACTTTGGATGGCAAAAAAGCCCTGTTGGATGTAGTTTCAG
AAAATGGGATAGAACACCGTATGGGAGTAGGAGTGCGC

>B.fragilis nanH gene_S6

CCGGTAGTTCAAGCAGCAGATACCATTTTTGTTCGTGAAACACGTATTCCTATTTTGATAGAAAGGCAGGA
TAATGTCTTATTCTATCTCCGTTTGGATGCTAAAGAGAGCCAGACCCTGAACGATGTAGTGTTAAACTTGG
GTGAAGGAGTGAATCTTTCTGAAATTCAGTCCATAAAGCTTTATTATGGAGGTACTGAAGCATTGCAGGAT

AGTGGTAAAAAACGTTTTGCTCCTGTTGGATATATTTCCAGTAACACTCCGGGGAAAACACTTGCCGCCAA
TCCGTCTTATTCAATCAAAAAATCAGAGGTGACTAATCCCGGTAATCAGGTTGTTCTGAAAGGAGATCAGA
AACTGTTTCCCGGAATCAATTATTTCTGGATTAGTTTGCAAATGAAGCCGGGCACTTCGCTTACCAGCAAG
GTGACCGCGGATATCGCTTCAATTACTTTGGATGGCAAAAAAGCCCTGTTGGATGTAGTTTCAGAAAATGG
GATAGAACACCGTATGGGAGTAGGAGTGCGC

>B.fragilis nanH gene_S7

TCCAGTAACACTCCGGGGAAAACACTTGCCGCCAATCCGTCTTATTCAATCAAAAAATCAGAGGTGACTAA
TCCCGGTAATCAGGTTGTTCTGAAAGGAGATCAGAAACTGTTTCCCGGAATCAATTATTTCTGGATTAGTT
TGCAAATGAAGCCGGGCACTTCGCTTACCAGCAAGGTGACCGCGGATATCGCTTCAATTACTTTGGATGGC
AAAAAAGCCCTGTTGGATGTAGTTTCAGAAAATGGGATAGAACACCGTATGGGAGTAGGAGTGCGCCATGC
CGGAGACGATAATTCTGCCGCATTCCGTATTCCGGGATTGGTGACTACTAATAAAGGTACGTTACTGGGAG
TTTATGATGTACGTTATAACAGTAGTGTTGACTTGCAGGAGCATGTTGATGTTGGTTTAAGCCGCAGTACA
GATGGTGGAAAAACTTGGGAAAAAATGCGTTTGCCTTTGGCTTTTGGAGAATTCGGTGGTTTGCCTGCCGG
TCAGAATGGGGTAGGA

>B.fragilis nanH gene_S8

ATTCGACACGTGTGCCCAATGCCGGTATTATGTACAGTAAAGATGGTGGCAAGAACTGGAAGATGCACAAT
TATGCACGTACGAACACCACAGAAGCCCAGGTAGCTGAGGTCGAACCCGGAGTGTTGATGTTGAATATGCG
TGATAATCGTGGAGGAAGCCGCGCTGTGGCTATTACAAAAGATTTGGGTAAAACATGGACGGAACATGAAT
CTTCTCGTAAGGCATTGCCGGAATCTGTTTGTATGGCTAGTTTGATCAGTGTGAAAGCAAAAGATAATGTG
TTGGGCAAGGATTTATTGATTTTCTCTAATCCTAATACGACAAAAGGACGCTATAATACTACTATTAAAAT
AAGTTTGGATGGCGGTGTGACTTGGTCACCCGAACATCAGCTGTTGTTGGATGAAGGCAATAACTGGGGTT
ATTCTTGCCTCTCTATGATTGATAAAGAGACGATTGGTATTCTTTATGAAAGTAGTGTGGCGCACATGACA
TTCCAGGCTG

DISCUSSION

Neuraminidase gene (*nanH*) is one of the genes that could play a role in the infection process encodes the *B. fragilis* neuraminidase enzyme (17), and this enzyme is it has been thought to promote virulence of *B. fragilis* by enhancing the ability of the organism to adhere, invade, and destroy the mucosal tissue (9). Molecular techniques such as sequencing of neuraminidase gene described in this study are promising tools for classification of these bacteria. However, basic information based, virulence and genetic compatibility is essential for clearer taxonomic definition of the *B.fragilis*.

A phylogenetic tree was inferred based on the *nanH* gene sequence of the Iraq isolates, and other *B.fragilis* available in GenBank. In the constructed tree, *B.fragilis* local isolates number (S1, S2, S3, and S7) were closely related to isolation *B. fragilis* recorded globally (M31663.1), and results of the analysis showed a clear convergence of isolates of *B.fragilis* local number (S5 and S6) to the isolation of *B.fragilis* recorded globally (D28493.1) and (Af031639.1) then this study recorded two unique local isolation of *B.fragilis* number (S4 and S8) differ from isolation recorded globally.

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