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Donnish Journal of Medicine and Medical Sciences
Vol 4(2) pp. 008-013 January, 2017.
http://www.donnishjournals.org/djmms
ISSN: 2984-8660

Original Research Article

Molecular Detection of Environmental *Morganella morganii* as Histamine Producing Bacteria

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Accepted 12th January, 2017.

Histamine-producing bacteria may be associated with much irritable bowel disease. The present study included fresh samples of Carp fish muscles for histamine producing bacteria (HPB) detection. Preliminary detection was depended on Niven´s medium, out of 25 bacteria samples, only 17 species were positive for histamine production. Molecular technique by PCR, the responsible Histidine – Decarboxylase (HDC) 709 pb was detected by specific primer. The results were compactable and parallel as those in Niven´s medium detected for histamine production. The bacterial species were identified by 16SrDNA sequence technique. The specimens (36) were diagnosed by amplifying universal primer of 16SrDNA. Sequence data were analyzed by (ncbi) program. Sequence results showed dominance of *Morganella morganii.*

Keywords: Histamine, *Morganella morganii*, 16S rDNA.

INTRODUCTION

Many bacteria species are known for decarboxylation of amino acid that produced histamine by their histadine – decarboxylase enzyme [1]. During or after fish poisoning, histamine producing bacteria may be introduced by contamination. Poisoning by histamine occurs through consumption of soiled fish with histamine [2,3]. *Enterobacteriaceae* is responsible for fish decomposition. *Morganell morganii* has been detected as histamine producer[4].

Geographical position season, water temperature, salinity and market environs, are factors that affect the composition and type of histamine-producing bacteria in fish or other food [5]. Many improved screening ways have been described for histamine producing bacteria detection [6,7,8,9]. Niven's medium [10] is widely used for (HPB). Recently, molecular methods were depended on to detect HPB in the environment and food samples.

Thousands of people each year suffer from food poisoning due to contaminated food and drinks with bacteria or viruses or toxins and may show symptoms such as high fever, diarrhea, vomiting and disturbances in the digestive tract. The foods that are often associated with food poisoning are meat, poultry, dairy products and unpasteurized dairy products, eggs, uncooked shellfish, fish and rice [11,12,13]. Microorganisms can produce high concentrations of biogenic amines by removing the carboxyl of free amino acids during the process of food spoilage and the concentrations of biogenic amines is used as an indicator of bacterial contamination in food. Biogenic amines are natural compounds which are produced during the metabolism of normal living cells and are also found in foods such as fish, cheese, wine, dairy products, meat and vegetables [14].

The responsible gene histidine decarboxylase enzyme is considered as a marker for histamine HPB detection. The present study is an attempt for the detection histamine producing bacteria in animal tissue by molecular methods.

MATERIAL AND METHODS

Sample collection

Fresh sample of Carp fish were gutted and then cut into small pieces. The sample was homogenized by hand shaking then diluted in sterile 0.1 % peptone [15]. The specimens were inoculated in duplicate on Trypticase soya agar (TSA) at 35°C for 24 hrs. Biochemical and Bacteriological the main test were depended on for bacterial diagnosis.

Histamine producing bacteria (HPB) detection

1. HPB detection by Niven's methods [10] one sample was inoculated on agar plate containing Niven's medium. The plate

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was inoculated at 37°C for 48hrs. [16]. Purple halo around Niven's medium is an indicator of histamine producing bacteria (HPB).

2. ***Molecular HPB deduction***

DNA extraction by kit as recommended by manual Kit instruction (According to Gene aid Kit).

Agarose gel preparation [17,18]

Reagents

* Buffer of 1ˣTBE
* Agarose
*Ethidium bromide
* Bromophenol blue

Agarose 0.2g was diluted by TBE buffer (25ml) then ethidium bromide (10 mg /ml) as 0.2 µl was added. The gel particles were dissolved by heating. After cooling the solution and the comb was instilled at one end, the gel tray was replaced in electrophoresis chamber**.**

DNA Loading and running

Bromophenol blue (3ml) was added to the DNA solution. The bands of DNA examined under UV.

Identification of HDCgene

The fragment of the *hdc* gene (709 bp) was amplified by [19,20]. Primers *f -hdc* (5´- TCH ATY ARY AAC TGY GGTGAC TGG RG -3´) and *r -hdc* (5´- CCC ACA KCA TBA RWG GDG TRT GRC C -3´). Twenty-five microliter of master mix PCR (50U/ml) *Taq* DNA polymerase, 400 µM of each of nucleotides (supplied by promega). Twenty nanograms of DNA and 75 Peg mole of reface and forward primers. The PCR processes were run for 40 cycles at 94°C for 1 min, 54 °C for 1 min and 72°C for 1 min (Theromcycal). Agarose 1% in 1ˣ TBE buffer (89 mM tris-borate, 2 mM EDTA, PH 8.3) at 86 V for 1 hrs. Ethidium bromide (0.3µg/ml) were added to gels to be visualized, burns and UV transit-laminate were used.

Bacterial identification (16S rDNA)

The bacteria specimens were diagnosed and detected by amplifying universal 16S rDNA by primers as shown in table (1-1).

Reagents

PCR amplification reagents are summarized in table (1-2).

PCR condition

PCR condition was described in table (1-3).

Electrophoresis

DNA and PCR product were detected by same procedure with some exceptions.

Electrophoresis running of 16S rDNA

DNA ladder (1Kb) as six ml and 5ml of 16S rDNA (PCR product) were run on 2% agarose gel for 1.5 h (60V). The bands were photographed under UV light by digital camera.

Sequencing of 16S rDNA

PCR product purification was adopted as recommended by Kit (Wizard Gel and PCR Clean-Up System). After above purification, final electrophoresis was run and single bands were obtained.

Sequencing of 16S rDNA 36 samples done at Eurofins MWG Operon BIONEER company http://eng.bioneer.com. The charging of samples was done as by Barker, M. [21].

Analysis of the sequence

Identification of bacteria species of the present study were dependent on analysis of 16S rDNA sequence data by program in the website http://www.Ncbi.nlm.nih.gov [12]. The sequence was aligned for the concatenated of 1327bp (the shorter sequence). The sequences product was copied and pasted in the species box. [22]. The bacteria species name will appear directly as percentage, 99% similarity or more were depended on for identification [23].

RESULTS AND DISCUSSION

Bacteriology investigation

The preliminary bacteriology investigation of fresh intestine of Carp fish samples were recorded . All bacterial species were Gram negative. The results were in agreement with many previous studies [24, 25, 26, 27], may be as a result of bile acids inhibitory effect on Gram-positive.

Histamine producing bacteria (HPB)

Purple halo around Niven´s medium is an indicator of positive HPB show figure (2-1) [14]. The table (2-1) show the bacterial samples that produce histamine than other bacteria. The Carp fish samples were not included G+ bacteria which explain the zero percent of positive HPB among G+. The above results may give us an explanation of many abdominal disturbances and irritable bowel disease among people that eat fish [28]. This abdominal disturbances are more frequent in consumption of spoiled fish that is called histamine poisoning [29,30,31]. Mlcneryey *et al*., (1996) claim very large amounts of free histidine found in many animal tissues.

The histidine is converted to histamine by decarboxylase enzyme [32]. Frozen fresh and tinned fish products that include high levels of histamine may be the main cause of fish histamine outbreaks [30]. Histamine levels more than 20ppm cause an adverse abdominal symptoms. The histamine levels (20-50ppm) considered as harmful as recommend by Food and Drug Administration (FDA) [33, 34]. Many research identified *Morganella morganii* that is isolated from fish as histamine producer [4], which agrees with the present study.

DNA extraction

The results of DNA extraction appear as bands show in figure (2-2).

Table 1-1: PCR amplification by universal 16S rDNA primer

Primers		Sequence	Length	Tm^	Optimizing TA^^
27	Forward	5´-AGAGTTTGATCCTGGC-3´	16	50.5	51.8°C
1492	Reverse	5´-GGTTACCTTGTTACGACTT-3´	19	42.8	51.8°C

Tm^: Melting temperature; TA^^: Annealing temperature

Table (1-2): Reagents of PCR amplification (50 µl) for 16S rDNA

No	Reagent	Amount
1	DNA template	10µl(30 ng)
2	Forward primer	2µl(20 pmol)
3	Reverse primer	2µl(20 pmol)
4	Taq Green Master Mix.2x	25µl
5	Nuclease-free Water	11µl
	Total volumes	50µl

Table 1-3: PCR amplification program for 16S rDNA

Steps	Temperature	Time	Cycle
Initial denaturation	92°C	2min	1
Denaturation	94°C	30sec	30
Annealing	51.8°C	45sec	
Extension	72°C	1.5min	
Final extension	72°C	5min	1

Table 2-1: Comparison histamine producing bacteria percentage among G^{-ve} and G^{+ve}

Type bacteria	HPB (%)	Non. HPB (%)
G^{-ve} Bacteria	17 (68%)	8 (32%)
G^{+ve} Bacteria	Non	Non

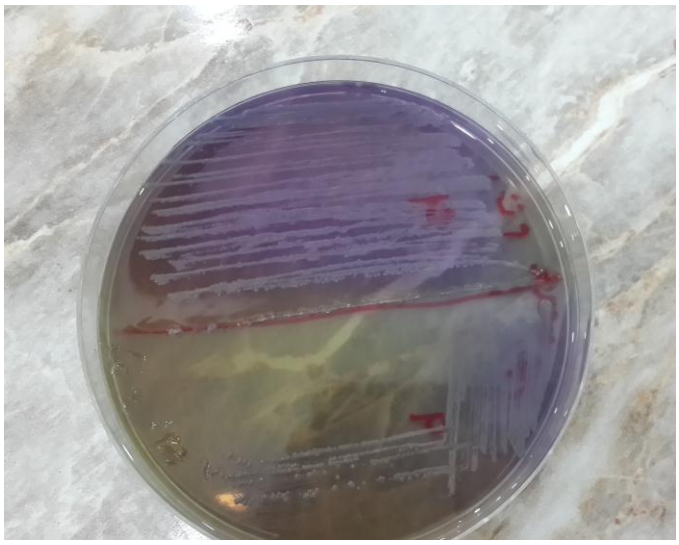

Figure 2-1: Purple halo around Niven´s medium is an indicator of positive HPB

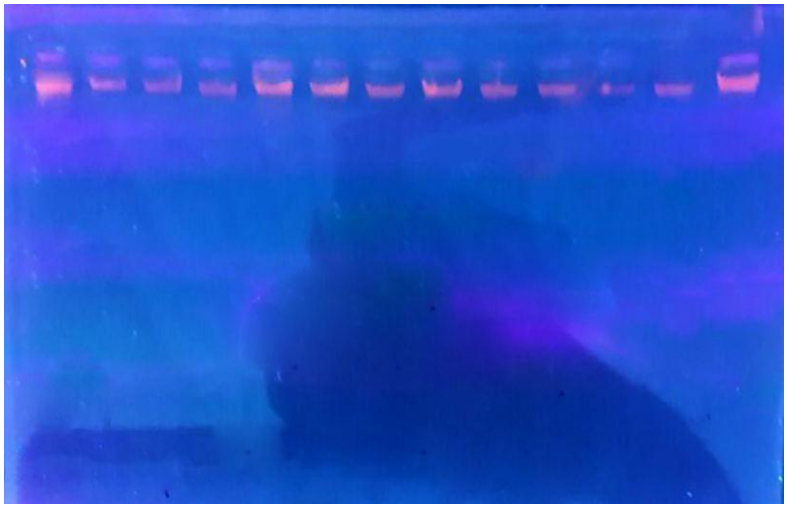

Figure 2-2: DNA extraction bands with ethidium bromide stain

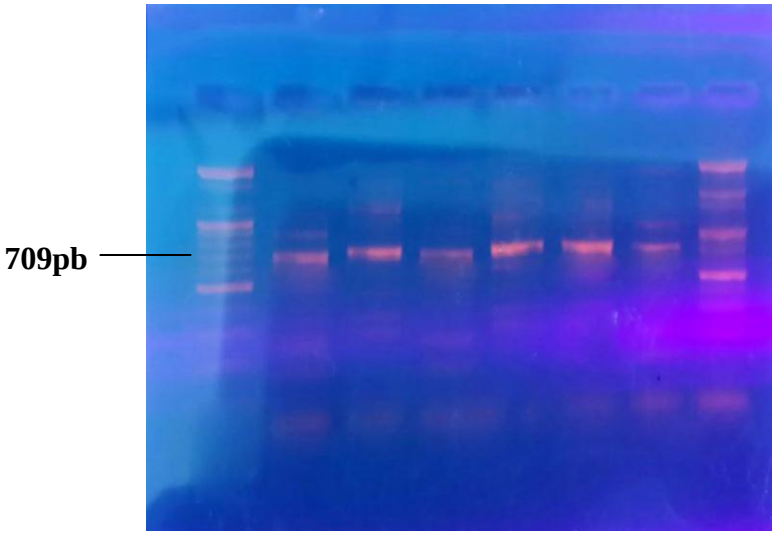

Figure 2-4: Positive Histamine producing bacteria for 709pb gene bands

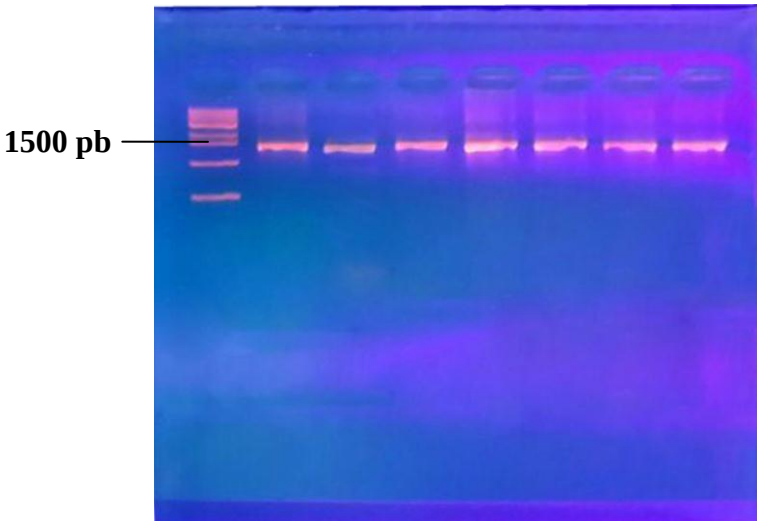

Figure 2-5: The bands of 16SrDNA gene of histamine forming bacteria

Table 2-3: The percentage of identical strain according to sequencing results

Bacterial isolates	Nucleotide Sequence	Source	Length (pb)	Identical To strain (%)
G-ve Bacteria	Fail	FM*	-	-
G-ve Bacteria	Read**	FM	1001bP	100%***Morganella morganii***
G –ve Bacteria	Fail	FM	-	-
G-ve Bacteria	Fail	FM	-	-

*FM fish muscle

**CAAAGTGGTAAGCGCCCTCCCGGAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGG
GAACGTATTCACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGTACT
TTATGAGTTCCGCTTGCCCTCGCGGGGTCGCTTCCCTTTGTATACGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTT
GACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCGCCATCACGCGCTGGCAACAAAGGATAAGGGTTGCGCTC
GTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTG
CTAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTC
ATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTC
GACATCGTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGC
CTTCGCCACCGGTATTCCTCCACATCTCTACGCATTTCACCGCTACACATGGAATTCTACCCCCCTCTACAAGACTCTAGCTGACCAGTATCAG
ATGCAATTCCCGGGTTAAGCCCGGGGATTTCACATCTGACTCAATCAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCA
CCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGTCGGTAACGTCAATTGCC

HDC gene results

The HDC genes were amplified by PCR technique and photographed by U.V. to detected 709bp gene as shown in figure (2-3). Seventeen bacterial isolates histamine producing were positive for 709pb gene bands. All bands were unique and distinguished PCR product. The results of hdc gene confirmed the causes of fish dietary tolerance for some people suffer from abdominal disturbances.

Bacterial identification by 16SrDNA gene

By PCR amplification, the sequence of 16SrDNA gene of histamine forming bacteria was detected figure (2-5). Bacterial identification has been a convenient way for accurate species identification. The dominance of confirmed histamine producing bacteria belongs to *Morganella morganii*. 16SrDNA gene sequencing results were summarized in table (2-3).

The table show nucleotide sequence of length (1385 pb) and identify percentage of strain under study as well as source of specimen. Out of seventeen sequencing, only five samples were sequenced, analyzed, identified and diagnosed as *Morganella morganii*. The results are an indicator for Enterobacteriaceae dominance than other bacterial groups which agree with many researches [35, 36, 37]. The sequence range of 16S rDNA nucleotides were ranged between (345-1350).

CONCLUSION

The present study may assist to avoid consumption of high histamine level that cause histamine food poisoning or food histamine intolerance. The present study suggests that further studies are necessary to detect the levels of histamine product in different foods. The further studies should include tissue and its normal bacteria flora.

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