Bacteriological Studies on Sea Turtles in Suez Governorate

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Abstract

Sea turtles are air-breathing, marine reptiles. The past few years have witnessed a surge in research focusing on the causes of morbidity and mortality, along with intensified endeavors to conserve them. This research aimed to isolate and characterize *vibrio alginolyticus* from apparently healthy sea turtles, Antimicrobial sensitivity test and Molecular identification. A total of 60 red eared turtle's samples (30 buccal and 30 cloacal samples),18 green turtle samples (9 buccal and 9 cloacal samples). collected aseptically from Red sea, Pet stores, Giza zoo and subjected to isolation and biochemical characterization of *V. alginolyticu, V. alginolyticus* are Gram negative motile comma shaped rodes that are facultatively anerobic. Isolates of *V. alginolyticus* divided into (9) positive isolates from Red eared turtle, (3) from Green turtle. All tested isolates were positive for catalase, oxidase, indole test, Methyl red and citrate utilization and Negative for Vogus proskeur. The *V. alginolyticus* strain exhibited major resistance to trimethoprim 5µg, ampicillin10µg, streptomycin 10µg based on the antibiotic sensitivity test, besides the intermediate sensitivity to Tobramycin 10µg, Naldixic acid 30µg and kanamycin (30µg). Furthermore, the bacterium demonstrated high sensitivity to Chloramphenicol 30µg, Tetracycline 30µg, and Ciprofloxacin 5µg. The molecular identification was achieved by utilizing species-specific primers to target the collagenase gene.

Keywords: *vibrio alginolyticus*, Turtle, antibiotic sensitivity test, collagenase gene, Biochemical Tests.

Introduction

Sea turtles, order Testudines, are marine reptiles. Recently, there has been a notable increase in the progress made in their medical care, research into the factors contributing to illness and death during stranding events, and conservation efforts aimed at safeguarding their populations. Sea turtles play important role in balance of ecosystem 1-maintain balanced food web (leatherback turtles consider as top jelly fish predators), 2-providing food for other animals (they present their bodies providing meals to shrimp and eager fish), 3-offering habitat (they serve as vital habitats for various marine organisms, particularly small creatures known as epibionts), 4- eating sea grass by green turtle's increase productivity and nutrient content of sea grass.

Within breeding centers and free-living turtles, bacterial infections typically manifest as a result of various infectious and environmental aspects (***Glazebrook and Campbell, 1990, Oros et al., 2005***). The presence of bacteria in the healthy turtles' natural microbiota could promote the development of diseases due to stress-inducing environmental factors and reduced immune system activity (***Mitchell and Kirchgessn, 2009).***

Numerous bacteria were recognized as the causative agents of diseases observed in captive marine turtles (***Chuen-Im et al., 2010***). Additionally, many of these bacteria have the potential to cause diseases in humans ***(Warwick et al., 2013)***. Bacteria such as *Aeromonas hydrophila, Pseudomonas fluorescens, V. alginolyticus, Bacillus spp.,* and *Flavobacterium spp.* frequently occur in sea turtles' bacterial microbiota of from Australia and Hawaii. They occur in association with other diseases, including fibropapillomatosis, obstructive rhinitis-pneumonia complex, and ulcerative stomatitis ***(Glazebrook and Campbell, 1990; Glazebrook et al., 1993; Aguirre et al., 1994)***. Vibrio spp., frequently occur in aquatic niches, can promote infections in humans ***(Chowdhury et al., 1989; West, 1989; Chakraborty et al., 1997).***

The successful identification and prompt diagnosis of bacterial diseases are of utmost importance. In the laboratory, molecular techniques employing the *16S rRNA* gene sequencing, in conjunction with biochemical characters, offer quick and precise technique for microbial identification ***(Buller, 2004).***

Material and method

1.Collection of Turtles samples:

A total of 60 samples collected from apparently healthy (30) Red eared Turtle and 18 samples from (9) Green Turtle collected from

(Red sea, Pet stores and Giza zoo). Samples were collected from Suez and Ismailia governorate, Buccal and cloacal samples were taken. Samples were transported to laboratory under aseptic condition for bacteriological examination as soon as possible.

2.Bacteriological examination:

Under strict aseptic conditions, samples were obtained from the mouth and cloaca using swabs, and then inoculated into TSB with NaCl (2%). Afterwards, isolates for *Vibrio* were inoculated into TCBS with NaCl (2%) and underwent incubation for 24-48hr at 25°c-28°c. When pure colonies have been grown, a loopful of each culture underwent streaking on slanted trypticase soya agar with NaCl (2%) to be used as a stock for subsequent biochemical identification. Isolates were preserved in BHI broth and TSA slant with 15% glycerol ***(Buller 2004)***. To identify the bacterial isolates biochemically, various tests, including Gram's stain, catalase, oxidase, and IMVC, were conducted.

3.Molecular identification and Partial sequences of 16SrRNA gene:

For genomic DNA extraction, Vibrio isolates underwent culturing on tryptic soya agar with NaCl (2%). For each sample, the PCR amplification reaction was accomplished in a 25 µl total volume of comprising: 4.5 µl PCR grade water, 12.5 µl 2X Dream Taq buffer, 6 µl Template DNA, and 1 µl of each primer (20 pmol). *Collagenase* gene was employed to specifically detect V. alginolyticus (***AbuElala et al., 2016)***.

The Oligonucleotide primers and PCR circumstances utilized in the study are described in (Tables 1,2).

4. Antimicrobial Susceptibility Testing

The determination of *V. alginolyticus* isolates antimicrobial susceptibility was carried out using the Kirby-Bauer disc diffusion technique. This involved utilizing (9) various antibiotics comprised ampicillin (10µg), streptomycin (10µg), tobramycin (30µg), Ciprofloxacin (5µg), trimethoprim 5µg , Naldixic acid 30µg, kanamycin 30µg, tetracycline 30µg, chloramphenicol 30µg. For each *V. alginolyticus* isolate, streaking was performed on Mueller-Hinton agar (Oxoid, England). Subsequently, antibiotic disks were placed on the plate, followed by incubation at 22°C for 24 hours. Measurements were taken of the inhibition zone diameters, and were subsequently interpreted following the ***CLSI (2006***).

Table (1)

Microorganism	Gene	Primer sequence (5'-3')	Length of amplified product	Reference
V. alginolyticus	*Collagenase*	CGAGTACAGTCACTTGAAAGCC CACAACAGAACTCGCGTTACC	737 bp	*Abu-Elala et al., 2016*

Table (2)

Target	Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
V. alginolyticus	*Collagenase*	94˚C/ 5 min.	94˚C/ 30 sec.	50˚C/ 40 sec.	72˚C/ 45 sec.	35	72˚C/ 10 min.

Results

1. Clinical examination

Atotal of 30 Red eared turtles and 9 green turtles from Red sea, Pet stores and Giza zoo. The clinical examination of collected samples revealed apparent healthy turtles with no clinical signs. Figure (1), (2), (3)

2. Bacterial isolation, prevalence of V. alginolyticus infection and phenotypic identification among examined sea turtles

yellow colonies of *V. alginolyticus* were recognized on TCBS agar, The bacterial isolates were Gram negative, motile comma shaped curved rods, oxidase and catalase positive, Prevalence of *V. alginolyticus* infection among the examined sea turtles was summarized in Table (3),(4)

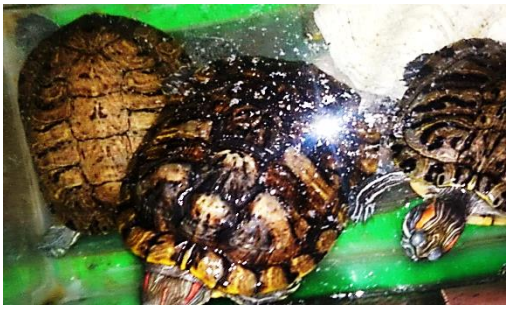
3.Molecular identification of *vibrio alginolyticus* isolates

Eight representive, biochemically confirmed *vibrio alginolyticus* isolates were identified by collagenase gene sequence Table (6) and Fig (4) illustrated the positive results for amplification of 737bp fragment of *vibrio alginolyticus* of collagenase gene for eight isolates from Turtles.

4.Antimicrobial susceptibility testing

Antimicrobial susceptibility investigation revealed that the *V. alginolyticus* isolates was highly resistant to trimethoprim, ampicillin, streptomycin and intermediate sensitivity to Tobramycin, Naldixic acid and kanamycin. The bacterium exhibited high sensitivity to chloramphenicol, tetracyclin and Ciprofloxacin.

(1)



(3)

Table (3): *V. alginolyticus* identification by biochemical testing:

Test	Reaction
Catalase	Positive
Oxidase	Positive
Methyl red	Positive
Indole	Positive
Citrate utilization	Positive
Vogus Proskauer	Negative
H2S production	Negative
Fermentation of lactose	Positive

Table (4): Frequency of *V. alginolyticus* infection within the inspected Sea Turtles:

Source	Number of Samples from each source			
	Red eared turtle		Green Turtle	
	Total	(+) v .alginolyticus	Total	(+) v. alginolyticus
Red sea	0	0	6	1
Pet stores	30	5	12	2
Giza zoo	30	4	0	0
		15%		16%

Table (5) distribution of *vibrio alginolyticus* in different organs of Sea turtles

	Species	No of isolates	Buccal sample		Cloacal sample	
Vibrio alginolyticus	**Red eared turtle**	9	4	44.4%	5	55.5%
	Green turtle	3	1	33.3%	2	66.6%
			41.6%		**63.6%**	

Table (6) genetic identification collagenase gene of vibrio alginolyticus isolates from Turtles.

Sample	0rigin of isolates	Vibrio alginolyticus collagenase gene
1	GreenTurtle	-
2	Red eared turtle	+
3	Red eared turtle	+
4	Red Eared Turtle	+
5	Green Turtle	+
6	Red eared turtle	+
7	Green Turtle	+
8	Red eared turtle	-

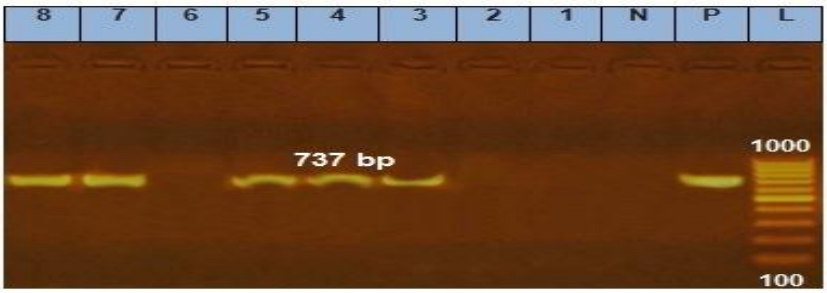


Fig (4): Agarose gel electrophoresis displaying the PCR findings for detecting *vibrio alginolyticus* collagenase gene representing amplification of 737 bp.
Lane L: 100-1000 bp DNA Ladder
Samples (3,4,5,7,8) V. alginolyticus gave positive reaction to collagenase gene.
Samples (1, 2, 6) gave negative reaction to collagenase gene.
Neg =control negative.
Pos = control positive

Discussion

V. alginolyticus has the ability to cause disease in numerous marine organisms like cnidarians, mollusks, fish, crustaceans, and sea turtles ***(Orós et al., 2004, 2005)***. In the present study, observed apparently healthy with no clinical signs sea turtles, which support the finding of ***Santoro et al. (2007)*** who reported that apparently healthy nesting green turtleswith no clinical signs of disease or external lesions were observed. TCBS agar, a selective medium, was employed for the bacteriological investigation of the isolated *Vibrio* species. The result noticed that *V.alginolyticus* exhibited sucrose fermentation, where yellow color colonies were detected. These outcomes agreed with findings of ***Hashem (2012), khalil (2014)***. Based on the biochemical identification, *V. alginolyticus* was found to be positive for catalase, methyl red, oxidase, citrate utilization, and indole, while it tested negative for H2S production and the Voges-Proskauer test (Tab 3). These results align with previous studies by ***Beleneva et al. (2004)*** and ***Abu-Elala et al. (2016)***. Using specific primers prepared for targeting the collagenase gene, the specific identification of V. alginolyticus was achieved. The PCR analysis yielded positive amplicons, which were recognized at 737 bp, as illustrated in Fig (4). These results align with the findings of ***Moustafa et al. (2015) and El-Hady et al. (2015)***. According to the antimicrobial susceptibility tests, *V. alginolyticus* isolates were highly resistant to trimethoprim, ampicillin, streptomycin and intermediate sensitivity to Tobramycin, Naldixic acid and kanamycin. The bacterium was proved to exhibit high sensitivity to Ciprofloxacin tetracycline, and chloramphenicol. Our results nearly agreed with ***Arafa et al. (2019) Yones et al. (2016), Elsayad et al. (2018)*** who documented the high sensitivity of *V. alginolyticus* to ciprofloxacin, tetracycline and chloramphenicol. The observed discrepancies in the antimicrobial sensitivities could be attributed to the significant rise in antimicrobial resistance. This highlights the urgent need to explore and develop new efficient antimicrobial agents ***(Abdallah2018).***

In conclusion this study is the first to profile bacterial pathogen in sea turtles in Egypt. this study provides a general picture of the prevalence and antimicrobial susceptibility profiling of *vibrio alginolyticus* in Red eared turtle and Green turtle. Vibrio *alginolyticus* exhibited variable level of antimicrobial resistance.this finding might be due to the wide use of antibiotics in aquatic environment.

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الملخص العربى

هذه الدراسة تلقي الضوء على دراسة الخواص الظاهرية والبيوكيمائية والجينية لعترات الفيبريو الجينوليتيكاس المعزولة من الترسة حمراء الاذن ومن الترسة الخضراء من حديقة الحيوان والبحر الاحمر ومتاجر الحيوانات الاليفة تم فحص عدد 39 ترسة بحرية (30) ترسة حمراء الاذن و(9) من الترسة الخضراء تم حصرها من البحر الاحمر محافظة السويس ومتاجر الحيوانات الاليفة وحديقة الحيوان. أظهر الفحص الظاهرى لانواع الترسة محل الدراسة عدم وجود اى اعراض مرضية ظاهرية. أخذت المسحات من الفم وفتحة الاخراج وتم تصنيف العينات المعزلة (الفيبريو الجينوليتيكاس) باستخدام وسائط مختلفة ونوعية واجراء اختبارات كيميائية حيوية. وقد تم فحص هذه العينات باختبارات بكتريولوجية لعزل ومعرفة ميكروب الفيبريو الجينوليتيكاس وقد اظهرت النتائج ان 9عينة معزولة من ترسة حمرا الاذن ايجابية الفيبريو الجينوليتيكاس بنسبة كلية 15% و3 عينات ايجابية الفيبريو الجينوليتيكاس فى الترسة الخضراء بنسبة 16% . فيما يتعلق بعينات الترسة كان عدد العينات الايجابية للفيبريو الجينوليتيكاس من العينات المأخوذة من الفم نسبة41.6 % فى كلا النوعين (الترسة الخضراء والترسة حمراء الاذن) بينما كانت نسبة العينات الايجابية من العينات الماخوذة من فتحة الاخراج 63.6%. تم عمل اختبارحساسية العترات للمضادات الحيوية المختلفة حيث أظهرت النتائج التي تم الحصول عليها أن العترات المعزولة من الفيبريو الجينوليتيكاس كانت عالية الحساسية للكلورامفينكول والسيبروفلوكساسين والتتراسيكلين بينما اظهرت مقاومة عالية للستربتوميسين والامبسيلين والتريمثوبريم .تم تأكيد العزلات على انها الفيبريو الجينوليتيكاس عن طريق تحليل تسلسل الكولجينيز جين