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EXPLORATION OF HAEMOLYTIC ACTIVITY AND ANTIBIOTIC SENSITIVITY OF BIOLUMINESCENT *VIBRIO* SPECIES FROM THOOTHUKUDI COAST

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ABSTRACT: Bioluminescence, the phenomenon of light emission by living organisms, is widespread in the marine environment at all the trophic levels from bacteria to fish and is thought to play a crucial role in the general ecology of marine plants and animals. Bacterial bioluminescence is very sensitive to toxic materials and hence, several investigations have tested its application for determining the toxicity of several chemicals. Although bacterial bioluminescence has found applications in various fields, there are certain reports where they have been found to be toxic to marine fishes and shrimps. The bioluminescent *Vibrio* sp. is especially noted to be pathogenic to shrimps causing necrosis and luminosis. Hence, the present study was carried out to determine whether the bioluminescent bacteria belonging to Vibrio sp. from Thoothukudi coast can lyse mammalian RBCs by haemolysis test and carry out the antibiotic sensitivity test of the isolated species using six antibiotics. Three different bioluminescent bacteria were isolated and identified as *Vibrio owensii*, *Vibro hyugaensis* and *Vibrio azureus*. *V. owensii* and *V. azureus* were γ-haemolytic and *V. hyugaensis* was α-haemolytic which meant that none of them were able to haemolyse the blood completely. 5µg and 10µg of Amoxicillin, Cefotaxime, Ciprofloxacin, Ofloxacin, Roxithromycin, and Tetracycline were used to carry out the antibiotic sensitivity test. *Vibrio owensii* was most sensitive, *Vibrio hyugaensis* was resistant, and *Vibrio azureus* showed intermediate results for the antibiotics used in the experiment. All the bacteria were resistant to Roxithromycin.

INTRODUCTION: Bioluminescence is a widespread phenomenon characterized by light emission produced in luciferase catalyzed oxidation of the substrate luciferin. Though bioluminescent bacteria are widely known for its luxuriant emission of natural light, they are also found to be the causative agents of various shrimp and fish diseases such as necrosis and luminosis.

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Bioluminescent *Vibrio* sp. are ubiquitous in seawater and apart from seawater, gravid female shrimps can discharge abundant colonies of luminous bacteria while captivity in hatchery [1, 2]. The natural habitat of *Vibrio harveyi* appears to be the gut of shrimps and marine sediments as to a free-living environment [3].

It has been characterized as an opportunistic pathogen causing disease in marine organisms [4]. Shrimps carrying luminous (51%) and non-luminous (46%) *Vibrio* sp. in the hatcheries of Taiwan were studied and correlated to the shrimps' death with the appearance of luminous *Vibrio* sp [5]. Luminous bacteria have been found to be the major problem in shrimp hatcheries of most Asian

countries [6]. The sensitivity of luminous *Vibrio harveyi* to nine selected antibiotics showed that the isolates were highly sensitive to gentamycin (10µg) and chloramphenicol (30µg), moderately sensitive to neomycin (30µg), and resistant to ampicillin (10µg), kannamycin (30µg), penicillin (10µg), streptomycin (10µg) and sulphadiazine (30µg) [7]. As various strains of luminescent *Vibrio* sp. are found to be pathogenic to shrimps and fishes and multi-resistant to antibiotics, the present study is carried out to check if the luminous bacteria of *Vibrio* sp. from Thoothukudi coast can be pathogenic to humans by carrying out haemolysis test and as a model to screen the various potential antibiotics that can evade them.

MATERIALS AND METHODS:

Collection of Samples: The marine water samples were collected from the Thoothukudi coast, Tamil Nadu, India. Sampling was done by taking proper aseptic measures and stored at 4 ºC for further use. The samples were processed for the isolation of marine luminescent bacteria.

Preparation of Luminescent Agar: For isolation purposes, optimization of media for luminescent bacterial growth is a very important parameter. A modified luminescent agar (LA) (Nealson, 1978) [8] was used in this study. The composition of Luminescent agar is as follows: 8g of dehydrated nutrient broth, 30g of Sodium chloride, 5g of Calcium carbonate, 15g of bacteriological agar, and 10 ml of Glycerol in IL of distilled water. The media was autoclaved at 121ºC for 15 min.

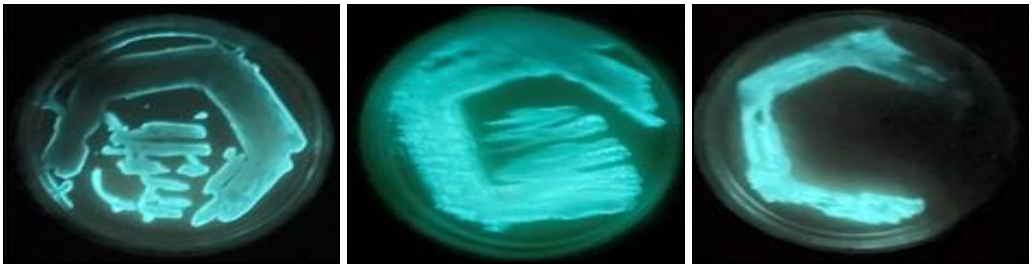
Isolation and Identification of Bioluminescent Bacteria: The collected marine water samples were added to the LA medium through pour plate method and incubated at about 20ºC for 24 h. Luminescent colonies were identified by observing the plates in a dark room. Three glowing colonies (LUM01, LUM02, and LUM03) were randomly picked up specifically and re-streaked onto LA plates and incubated at 20ºC for 24 h. They were further purified by sub-culturing in luminescent agar plates following standard bacterial isolation by repeated streaking on luminescent agar and stored in the refrigerator for further use. The isolated colonies were identified by 16srRNA sequencing.

Haemolysis Test: Haemolytic activity of the isolated luminescent strains (LUM01, LUM02, and LUM03) was carried out by streaking them in Sheep Blood Agar plates and incubated at 28ºC for 48 h. Haemolytic activity was detected by the presence of a definite, clear zone around the colonies indicating complete haemolysis or by the appearance of greenish colonies indicating partial haemolysis. The absence of zones and colouration indicates negative haemolysis.

Antibiotic Sensitivity Test: Six different antibiotics, namely Amoxicillin, Cefotaxime, Ciprofloxacin, Ofloxacin, Roxithromycin, and Tetracycline were used in two different concentrations: 5µg/disc and 10µg/ disc. The luminescent strains were swabbed uniformly onto the LA agar plates, and the discs containing the different concentrations of antibiotics were placed on the plates along with a control. The obtained zones of diameter were measured in mm and were classified into Resistant (0mm – 3mm), Intermediate (4mm-8mm), and Sensitive (9mm-14mm).

RESULTS AND DISCUSSION:

Isolation and Identification of Bacteria: Three specific colonies were selected by observing their luminescence in the dark. The isolated bioluminescent colonies were identified as *Vibrio owensii* (LUM01), *Vibrio hyuganesis* (LUM02), and *Vibrio azureus* (LUM03) by 16srRNA sequencing **Fig. 1.**



VIBRIO OWENSII ***VIBRIO HYUGANESIS*** ***VIBRIO AZUREUS***

FIG. 1: BIOLUMINESCENT VIBRIO SP. ISOLATED FROM THOOTHUKUDI COAST

Haemolysis Test: *Vibrio owensii* and *Vibrio azureus* showed negative haemolysis as there was no formation of a clear zone or the appearance of greenish colour. This is due to the fact that the bacteria do not produce any toxins that can lyse the red blood cells. They lack haemolysis and are said to be γ haemolytic. *Vibrio hyuganesis* showed greenish color around the colonies, and hence it is α haemolytic. This means that the bacterium is able to reduce haemoglobin into methemoglobin which is only partial lysis of the red blood cells **Fig. 2.**

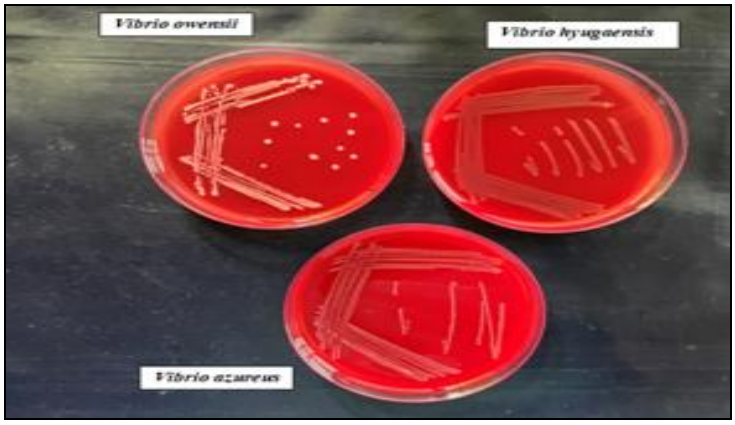


FIG. 2: HEMOLYSIS TEST OF THE ISOLATED BACTERIA IN SHEEP BLOOD AGAR PLATES

Antibiotic Sensitivity Test: All the bioluminescent bacteria showed resistance to Roxithromycin. *Vibrio owensii* was most sensitive to Ofloxacin with a zone of 14mm at 10µg concentration. *Vibrio hyugaensis* was highly sensitive to Ofloxacin and Ciprofloxacin, each with a zone of 10mm at 10µg concentration. *Vibrio azureus* was highly sensitive to Tetracycline with a zone of 10mm at 10µg concentration. *Vibrio azureus* did not show much difference in the diameter of zones between 5µg and 10µg concentration in all the antibiotics **Fig. 3** & **Table 1**.

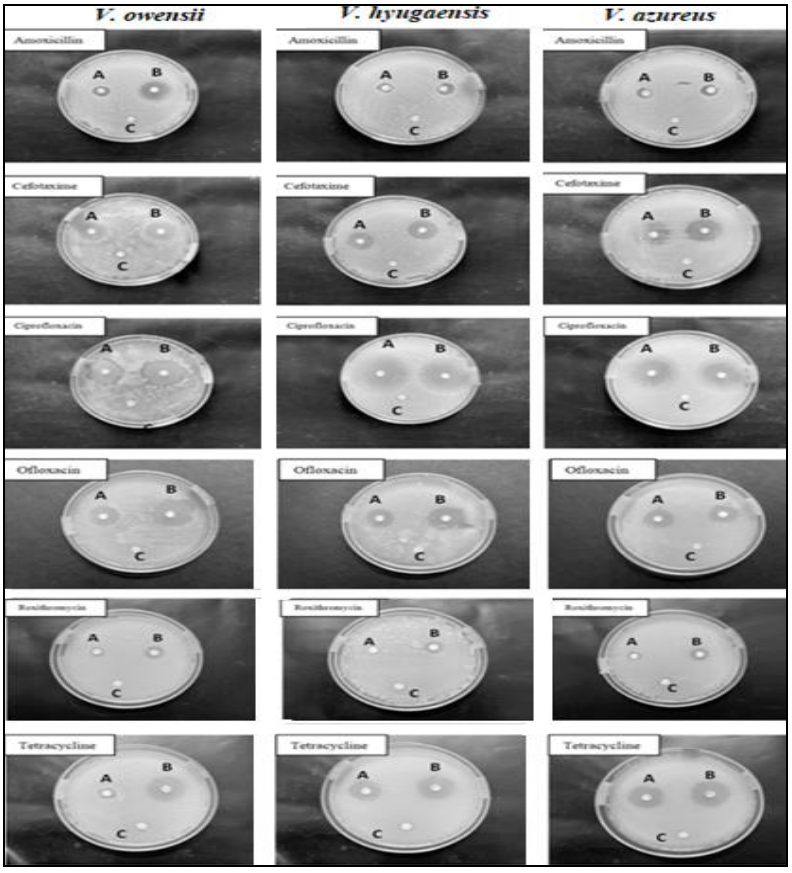


FIG. 3: ZONE OF DIAMETER OBTAINED IN ANTIBIOTIC SENSITIVITY TEST A-5µg B –10µg C- CONTROL

TABLE 1: ZONES OF DIAMETER IN MM OBTAINED THROUGH ANTIBIOTIC SENSITIVITY TEST

Bioluminescent bacteria	Amoxicillin		Cefotaxime		Ciprofloxacin		Ofloxacin		Roxithromycin		Tetracycline	
	5µg	10 µg	5µg	10 µg	5µg	10 µg	5µg	10 µg	5µg	10 µg	5µg	10 µg
V. owensii	4(I)	8(I)	7(I)	9(S)	10(S)	12(S)	7(I)	14(S)	1(R)	3(R)	3(R)	10(S)
V. hyugaensis	2(R)	3(R)	5(I)	7(I)	10(S)	10(S)	6(I)	10(S)	0(R)	2(R)	4(I)	8(I)
V. azureus	3(R)	4(I)	5(I)	8(I)	8(I)	8(I)	5(I)	6(I)	2(R)	4(I)	7(I)	10(S)

All the values are expressed in mm. S- sensitive, I- intermediate and R- resistant.

DISCUSSION: Microbial inspection of α-haemolysed red blood cells shows that the cell membrane remains intact; hence it is not true lysis. The greenish appearance of the colonies can be equated to bruising the cells. In contrast to the present investigation, it is reported that about twelve luminous *Vibrio* strains isolated from Snook cornea and aquarium water have shown 100% β-haemolytic activity of sheep RBC [9].

The antibiotic sensitivity tests showed that the diameter of the zones increased with the increase in concentration for almost all the bacteria. The luminescence of the bacteria disappeared with the addition of antibiotics discs. The loss of luminescence is due to hereditary influence. Temporary loss of luminescence can be caused by modifying the media or adding various substances [10].

Weakening in luminosity is always accompanied by an arrest in growth. The expression or absence of luminescence in the presence of the antibiotics indicates each antibiotic's toxicity level towards the luminescent isolate [11].

On the contrary, about thirteen cultures of luminescent *Vibrio* sp. showed Multiple Drug Resistance (MDR) for Tetracycline, Ampicillin, Gentamycin, Penicillin, and Ciprofloxacin [12].

The *lux* genes of the bioluminescent bacteria have found applications as a tool for a biosensor of pollutants, marker gene for diagnosis, and for food industries for detection of contaminants. The pathogenicity of the luminous bacteria must be studied to avoid replication of strains in becoming multi-resistant to antibiotics as they have numerous advantages in screening heavy metal pollutants.

CONCLUSION: Bioluminescent bacteria belonging to *Vibrio* species are excellent biosensors of environmental pollutants. Though these species are highly recognized for their potential to detect heavy metals, they are also noted to be causing diseases in aquatic animals. Hence the present study was carried out to analyze the antibiotic sensitivity of the Vibrio species against various antibiotics for future reference and to check its effect on mammalian RBC.

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CONFLICTS OF INTEREST: The authors do not have any conflicts of interest.

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