

## Immunological Evaluations in Mucosal Buccal Polyps of *Nemipterus japonicus*: An In-Vitro Study

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**Abstract:** Immunity is an ecologically relevant trait, which is of key importance for organism survival and population growth against the pressure of pathogens in their environment. The aim of this study is to evaluate the pathogenicity of *Nemipterus japonicus* due to bacterial species and metal exposures. In the present study, a series of serological tests were conducted against pathogens and metal pollutants isolated from buccal mucosal polyps of *Nemipterus japonicus*. Agglutination test revealed visible clumps within a few seconds after addition of antiserum of *Klebsiella pneumoniae*. The number of lymphocytes present in the culture is determined by using haemocytometer and stimulative index is calculated to find out lymphocyte proliferation in pathogenic induced study. *Klebsiella pneumoniae* has a comparatively high lymphocyte stimulative index than *Vibrio vulnificus* and *Aeromonas hydrophila*. In metal induced study, elevated stimulative index of lymphocytes was observed in Lead (Pb) and Chromium (Cr) than Cobalt (Co), Copper (Cu), Zinc (Zn) and Mercury (Hg). Out of fifty samples tested, forty two (94%) were positive for *Klebsiella pneumoniae* antibodies in significant titre. An index value was calculated to generate the results for either IgG or IgM as follows: negative, < 9; equivocal, 9 to 11; and positive, > 11.

**Keywords:** *Nemipterus japonicus*, Polyp, *Klebsiella pneumoniae*, ELISA, IgG, IgM

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### I. Introduction

The immune system of fishes is often considered to be a primitive one. This is a system of biological structures and processes within an organism that protects against disease. Health of fish depends on the interrelationship of some major components of the fish, and the environment in which they live. The environment may be the most critical component of the fish health matrix because environmental quality influences the fish's physiological well-being, species cultured, feeding regimes, rate of growth, and ability to maintain natural and acquired resistance and immunity. Given the importance of the immune system for fitness and ecology of organisms, it is evident that a possible disrupting impact of environmental chemicals on the immune system may have far-reaching consequences. In fact, a wide variety of chemicals has been reported to impact immune parameters of teleost fishes (1).

Japanese thread fin bream, *Nemipterus japonicus* is a demersal species and widespread in the Red Sea and eastern shores of Africa to the Philippines and Japan (2). A specimen of the Japanese thread fin bream *N. japonicus* was recorded for the first time from the Mediterranean Sea by (3) and then (4). The catch ratio of *N. japonicus* has been increased within last decade and become as one of the main commercial species. Biology of *N. japonicus* has been studied by various authors in different regions (5, 6 & 7) and many others in India (8). Large number of *N. japonicus* in the coastal area of Visakhapatnam is found to be suffering from mucosal buccal polyps and other associated manifestations. There are many reports on the biological aspects of Nemipterids, but very few on the effect of pollution and parasite infections (9, 10, 11, 12, 13, 14 & 15). The present study aims to screen the potential pathogen and marine water pollutant of mucosal buccal polyps of *N. japonicus* using serological assays.

### II. Research Methodology

**2.1. Sample collection:** The blood was collected on board from polyp presented or diseased *Nemipterus japonicus* and healthy *Nemipterus japonicus* as a control by making an incision at the caudal end, using 50ml hypodermic syringe with 26G needle. Blood samples were allowed to clot and further serum was separated by centrifugation for 20 minutes at 3000 rpm at room temperature. The diagnosis of *Klebsiella* infection is made on the basis of clinical examination, isolation and identification of the organism (16). Laboratory identification is usually carried out by cultures, gram staining and a battery of biochemical tests and serological findings like agglutination, Lymphocyte stimulation test in detection of immune stimulatory effect of pathogen, metals MELISA and ELISA. Bacterial isolation takes 24 hours and serological tests have their own limitations in that the results may be available only after 48 hours (17).

## 2.2. Agglutination test

The slide agglutination tests were performed for all isolates to confirm their identity (18). Each isolated species of *Klebsiella pneumoniae* was emulsified in a drop of normal saline placed in a ringed slide. Then one drop of *Klebsiella pneumoniae* antiserum is added to the emulsion of the organism already made on the slide and gently rotated. Appearance of visible clumps within a few seconds denotes positive agglutination. A control saline emulsion of the organism is also made in a separate ring. The control did not show any agglutination.

## 2.3. Lymphocyte stimulation test in detection of immune stimulatory effect of pathogen

To 2.5ml of RPMI 1640 complete medium with antibiotics (10,000 U/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate) 1X was taken in centrifuge tubes, to it 250 µl of heparinised blood and 50µl of PHA (10µl /ml) was added and incubated for 72 hrs at 37°C. At the end of the incubation 100µl of antigen is added. The solution was centrifuged and the pellet obtained is washed twice and incubated. The total number of lymphocytes measured microscopically using haemocytometer. A stimulation index was calculated as follows

SI= Total number of WBC in pathogen proliferated blood sample/ number of lymphocytes in control

## 2.4. Lymphocyte stimulation test in detection of immune stimulatory effect of metals

To 2.5ml of RPMI 1640 complete medium with antibiotics (10,000 U/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate) 1X was taken in centrifuge tubes, to it 250 µl of heparinised blood and 50µl of PHA (10µl /ml) was added and incubated for 72 hrs at 37°C. At the end of the incubation 100µl metal solutions (Cobalt: 15ppm, Copper: 2.5ppm, Zinc: 20ppm, Lead: 10ppm and Mercury: 0.25ppm) added separately for each sample. Incubated for 72 hr at 37°C in a cell culture incubator. Flasks were kept upright with caps closed. This method helps to visualize the normal lymphocytes from transformed cells and also the stimulated WBC from unstimulated WBC.

### 2.4.1. Differential count of white blood cells

The smear was stained with 7-10 drops of leishman's stain and allowed to stand for 5 minutes. At the end of the incubation phosphate buffer was added for about 10-15 drops and is allowed to stand for 20 minutes and then washed with flowing water to remove the excess stain. The slide was air dried and observed under microscope.

### 2.4.2. Total count of white blood cells

The blood sample was drawn into the WBC diluting pipette and diluted with WBC diluting fluid. Blood and the WBC diluting fluid mixed for about 3 minutes. Neubauer Chamber was charged with this fluid and a cover slip was placed on it and observed under microscope.

### Calculation

Total number of WBC observed = n

$$\text{WBC/mm}^3 = \text{Total number of WBC observed} \times \text{Dilution factor} \times \text{Depth of chamber} \times \text{Number of squares.}$$

$$\text{WBC/mm}^3 = n \times 20 \times 10 \times 4$$

## 2.5. ELISA (Pan-Bio)

An enzyme linked immunosorbent assay (ELISA), was adapted to detect antibodies against *Klebsiella pneumoniae* in *N. japonicus*. IgM ELISA using *Klebsiella pneumoniae* antigen attached to polystyrene surface of micro well test strips was performed according to manufacturer's instructions employing 10 µL of fish serum sample (19). After addition of serum, plates were incubated for 30 min at 37°C. HRP (Horseradish peroxidase) conjugated anti-sera IgM and TMB (3, 3', 5, 5'-Tetramethylbenzidine) (20) were provided by the manufacturer and they were used as per instructions. Then the plate was read at OD<sub>450</sub> nm and the readings were interpreted in terms of Pan-Bio units calculated as per manufacturer's instructions. Positive control serum, negative control serum and cut-off calibrator, provided by the manufacturer were used for calculation of Pan-Bio units from the observed absorbance. Each runs included positive, negative, and cut-off calibrator controls.

### III. Results

#### 3.1. Agglutination

Visible clumps were observed within a few seconds after addition of antiserum of *K. pneumoniae* and no clumps were observed in the control samples. Negative results or no clumps were observed after addition of antiserum of *V. vulnificus* and *A. hydrophila*, the emulsion remains as it is.

#### 3.2. Lymphocyte stimulation test in detection of immune stimulatory effect of pathogen

The number of lymphocytes present in the culture is determined by using haemocytometer and stimulative index is calculated to find out lymphocyte proliferation to different pathogens.

#### 3.3. Lymphocyte stimulation test in detection of immune stimulatory effect of metals

The number of lymphocytes present in the culture is determined by using haemocytometer and stimulative index is calculated to find out lymphocyte proliferation to different metals.

#### 3.4. ELISA Test

Fifty serum samples were tested for *Klebsiella pneumoniae* antibodies by ELISA test. Out of fifty samples tested, forty two (94%) were positive for *Klebsiella pneumoniae* antibodies in significant titre. This sampling is limited in view of non availability of sufficient ELISA kits which has to be imported. An index value (PANBIO units) was calculated to generate the results for either IgG or IgM as follows: negative, < 9; equivocal, 9 to 11; and positive, >11. The ELISAs could be completed in around 2.5 h.

### IV. Discussion

Serology is an alternative approach to pathogen detection, and can also be applied to the detection of pathogen-specific antibodies in fish. A number of new technologies are being developed for the rapid detection of pathogens and monitoring host responses. Therefore, it can be used in molecular and immunodiagnosics to detect pathogens directly from tissue samples or culture, or it can be used in serology to measure fish antibodies (21 & 22). In the present study mucosal buccal polyps of *N. japonicus* was screened for bacterial pathogens and metal pollutants by various serological tests.

Microbes are a part of the natural flora of coastal marine environments worldwide and has been isolated from water, sediments, and a variety of seafood, including shrimp, fish, oysters, and clams (24, 25, 26 & 27). In the present study, infected *Nemipterus japonicus* collected from Bay of Bengal waters. As Nemipterids are bottom dwellers, these fish are mostly affected by the polluted environment in which they live. These demersal species are particularly susceptible to physical abnormalities and diseases, which appear to be associated with contaminated sediments (28).

Our study revealed, significant bacterial loads in the mucosal buccal polyps of *N. japonicus*. Since mucus surfaces are important defense barriers against bacterial infection, mucus layers in fish are related to a number of activities including the prevention of colonization by pathogen. These observations are supported by many scientists.

In the present study the fish collected from marine water of Bay of Bengal showed, mucosal buccal polyps. Microbial and serological investigations were conducted on these mucosal buccal polyps. Microbiological studies revealed that *Klebsiella pneumoniae* infection in *N. japonicus* is the most common pathogen which develops polyps. Along with *K. pneumoniae*, *V. vulnificus* and *A. hydrophila* were observed in some infected fishes. But *K. pneumoniae* is major pathogen, which showed significant load and is correlated with the earlier studies made by several investigators (15, 29, 30 & 31).

On the other hand in our study, we observed significant increases of Lead and Chromium in marine water. Lead (Pb) is ubiquitous toxic contaminant in our environment which poses a substantial threat to animal and human health. There is plenty of evidence that lead is very genotoxic (34, 36), but some studies have focused on the possible immunotoxic effects of lead (32, 33, 35, 37, & 38). Lymphocytes treated with lead showed increased proliferation of non-infected cells, but significantly suppressed the proliferation of Bovine Leukaemia Virus (BLV) infected cells. Activity of the DNA polymerases decreased proportionally to lead concentration in all BLV-infected lymphocyte cultures (39). The results suggest that the metals may affect the lymphocyte directly by altering the synthesis of cellular DNA and thereby influence the immune responses.

Metal in the aquatic environment are bioaccumulated by organisms either passively from water or by facilitated uptake. Metal ions can penetrate inside the cell, interrupting cellular metabolism and in some cases can enter the nucleus. Metal cations can bind to DNA through ionic and coordinated bonds in a reversible way, but cannot produce all the lesions observed in chromatin of cells. Hence, not only the direct, but mostly indirect effects of metals on nuclear chromatin must be considered more important in DNA damage (40). Metal distribution between the different tissues within an organism depends on the mode of exposure and can serve as

pollution indicator. Few scientists have reported metal accumulation in *Nemipterus* species. (41) reported on the concentration of Fe, Co, Ni, Cu, Zn and Pb in the muscle tissue of *N. japonicus* from Kochi and Mangalore on South West Coast of India. Though our study suggested that *K. pneumoniae* is indeed the primary etiological agent of buccal mucosal polyps, even metal pollutants may involve in the development of other pathological findings (42, 43 & 44).

### V. Conclusion

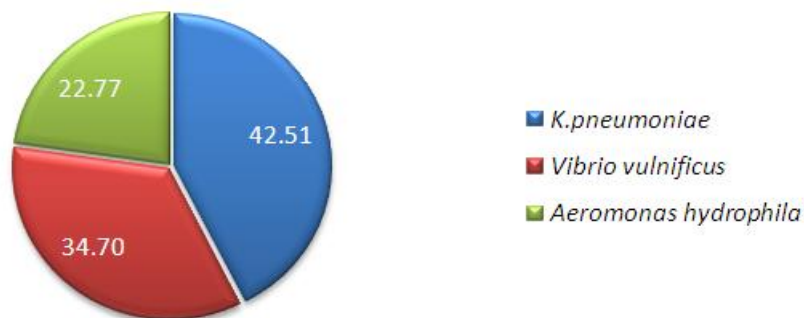
This study focuses on the importance of immune system for organism fitness and population growth, in both their environment and constant exposure to a wide range of metal pollutants and pathogens. As the immune system is the key success parameter in the “race of arms” between pathogens and hosts, this study intends to highlight, that the immune system is regulated in a multifactorial way.

### Acknowledgements

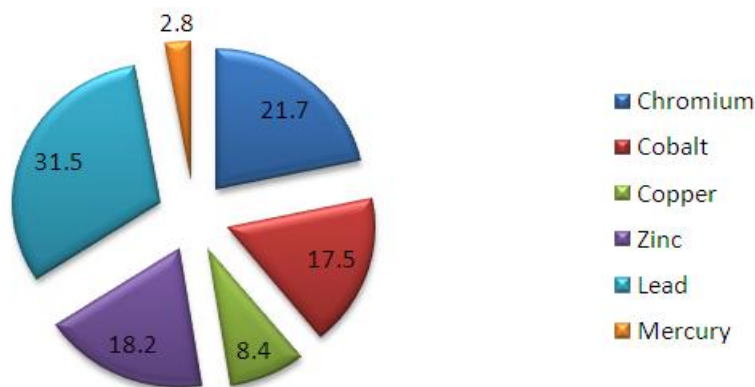
The first author expresses her gratitude to the Department of Science and Technology (DST), government of India, New Delhi for giving financial support under the women scientist scheme- A.



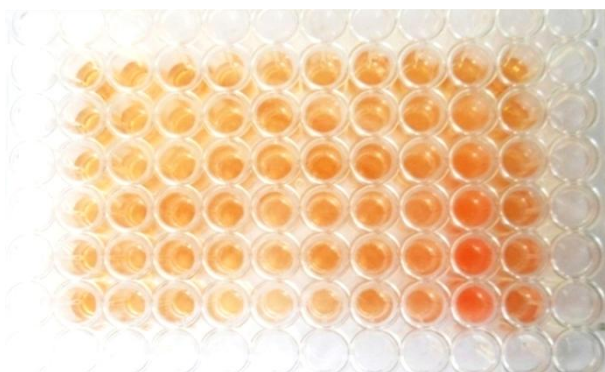
**Fig.1** Polypoid mass in the buccal cavity



**Fig-2** represents *Klebsiella pneumoniae* has a comparatively high lymphocyte stimulative index than *Vibrio vulnificus* and *Aeromonas hydrophila*



**Fig-3** represents an elevated stimulative index is observed in Lead (Pb) and Chromium induced lymphocyte count compared with other metals.



**Fig: 4** represents Enzyme-linked immunosorbent assay (ELISA) for *K. pneumoniae* antibody (immunoglobulin) in a 96-well

**Table: 1** shows WBC count in lymphocyte culture

Parameters	Units	Control	<i>K. pneumoniae</i>	<i>Vibrio vulnificus</i>	<i>Aeromonas hydrophila</i>
WBC	Cells/ cu	0.5±0.1	3.92±0.7	3.2±0.31	2.1±0.6

\*All the mean values of ten determinates

**Table: 2** shows experimental Results Lymphocyte stimulative index for metals

Parameter	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6
Control	1.5±0.1	1.5	1.5	1.5	1.5	1.5
Chromium(10 ppm)	3.1±0.8	-	-	-	-	-
Cobalt(15 ppm)	-	2.5±0.4	-	-	-	-
Copper (2.5ppm)	-	-	1.2±0.9	-	-	-
Zinc (20ppm)	-	-	-	2.6±0.4	-	-
Lead (5 ppm)	-	-	-	-	4.5 ±0.2	-
Mercury (0.5ppm)	-	-	-	-	-	0.4±0.1

\*All the mean values of ten determinates

**Table: 3** shows the dilutions of metals for ELISA

Metal	Control	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6
RPMI (ml)	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
Heparinised blood (ml)	250µl	250µl	250µl	250µl	250µl	250µl	250µl
PHA (ml)	50µl	50µl	50µl	50µl	50µl	50µl	50µl
Chromium(10 ppm)	-	100µl	-	-	-	-	-
Cobalt(15 ppm)	-	-	100µl	-	-	-	-
Copper (2.5ppm)	-	-	-	100µl	-	-	-
Zinc (20ppm)	-	-	-	-	100µl	-	-
Lead (5 ppm)	-	-	-	-	-	100µl	-
Mercury (0.5ppm)	-	-	-	-	-	-	100µl

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