MICROFAUNAL DIVERSITY AND POPULATION DYNAMICS OF HYPERPARASITE INHABITING FISHES OF GOMTI RIVER AT LUCKNOW

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SUMMARY

It is one of the major challenges to predict and control the infectious disease prevailing in humans, wildlife sustainability, fishery, and agriculture. Parasites of fishes may also be infected with hyperparasites (parasites whose host itself is a parasite). Most cases of hyperparasitism have been found in insects, but fish hyperparasitism remains very rare yet influential. Hyperparasites can severely impact pathogen density and pathogen infection dynamics. Hyperparasites can be used as a biocontrol agent. Infection of hyperparasites may control the virulence of parasites. When hyperparasites induce hypo-virulence, they can limit both the severity and transmission of infectious diseases (Parratt and Laine, 2016). Studies related to hyperparasitism are scarce in India. The test for the local adaptation revealed that hyperparasites were more successful at infecting sympatric pathogen strains than allopatric ones (Parratt et al., 2016).

Several cases of hyperparasitism of helminth on helminths believe that hyperparasitism is the result of intraspecific competition for space, when intensities of parasite infestation are high (Sey and Moravec, 1986). Hyperparasitism on trematodes was first described in the late 19th–early 20th century (Dugarov et al., 2011). Protozoan hyperparasites, *Urosporidium spisuli* reported in surf clams (*Spisula solidissima)* infected with anisakid nematode worm from Virginia and North Carolina (Perkins et al., 1975). During a survey of parasites of fishes, *Nosema aegyptii* (microsporidia) was found for the first time in the coelomic cavity and musculature of *Procamallanus elatensis. Nosema aegyptii* was observed in one of the nematodes removed from the intestine of siganid fishes (Abdou and Heckmann 2000). Most of the cases of hyperparasitism in fishes take place by protozoan parasites, but a number of helminth parasites are reported which are parasites of other helminths.

Microsporidian infection in *Liza ramanda* (Risso) from Bardowil Lagoon, Mediterranean coast of Sinai in fibroblast of the metacercarial cyst. Infection of metacercarial cyst resulted in hypertrophy of the cyst wall and degeneration and eventual death of the encapsulated metacercariae (Paperna et.al., 1978). Three myxosporeans and two hyperparasitic microspores were reported in the intestine of an emaciated cultured tiger puffer (Tun et al., 2000). Two unusual cases of hyperparasitism of trichodinid ciliates were reported on monogenean flukes. The first case was observed in March 2001 in a concrete pond holding approximately 30000 European sea bass *Dicentrarchus labrox* of about 200g, whose gills were found to be heavily infected by a trichodinid and moderately infected by *Diplectanum aequans.* The second case was observed in March 2001, in one individual devil fire fish (Lion fish) *Pterois miles* collected in the shallow waters of Eilat's North Beach (Colorni and Diamant, 2005).

During the study of the parasite fauna of the European eel in northwest Spain, mixed infection of branchial tissues by the myxosporidium *Myxidium giardii* and by two monogeneans (*Psuedodactylogyrus anguillae* and *Psuedodactylogyrus bini*) were reported (Aguilar et al., 2004). Phylogeny of the microsporidean hyperparasite infecting *Marteilia cochilia* in cockles and a further hyperparasite *Unikaryon legeri* infecting the digenean infecting the *Meigymnophallus minutus,* was investigated. They showed that rather than representing basally branching taxa in the increasingly Cryptomycota/Rozellomycota out group, the hyperparasite instead group with microsporidean parasites infecting aquatic crustaceans (Stentiford et. al., 2011). Climate change also plays a major role in fish-parasite interaction. Due to variations in seasons and temperature, parasites and their host physiology were affected. Parasite prevalence may increase or decrease with a change in temperature (Lohmus and Björklund, 2015).

The objectives of the present research work were to study the population dynamics of parasites and hyperparasites of fishes and identify

the parasites and hyperparasites on the basis of morphology, biochemical test, molecular characterization of hyperparasites of fishes, and finally to assess the seasonal variation in parasites and hyperparasites of fishes. To accomplish all these objectives, firstly fish samples were collected from the Gomti River and parasites were isolated after the dissection of fishes. The research site was the Gomti River in Lucknow, Uttar Pradesh. The work was accomplished over the duration of a year, from March 2019 to February 2020. In the early stages of my investigation, I visited three locations at the Gomti River: Pakka Pul, Mehndi Ghat, and Ghaila Bridge. Out of these three locations, Ghaila Bridge was selected. Ghaila Bridge is located 22 kilometers to the northeast of the BBAU Campus at 29° 90' 22"N and 80° 87' 46"E. Fish from the Gomti River at the Ghaila Bridge, particularly, are observed to be preferable to get in live condition, achieving the goal of getting live parasites with the greatest likelihood.

Various physicochemical characteristics, including water temperature and pH, were measured in water samples taken from the Ghaila bridge site of the Gomti River. The obtained samples were examined for various physicochemical characteristics in accordance with APHA (2005). Using a mercury-filled thermometer, the temperature was measured, and the findings were given in degrees Celsius. Using a conductivity meter, the pH of the water was measured.

Three different fishes were selected namely- *Channa punctatus*- Common name- Girai; *Heteropneutes fossilis*- Common name- Singhi; *Rita Rita*- Common name- Rita. Fish samples were obtained with the assistance of experienced local fishermen near the Gomti River's Ghaila Bridge.
Fish endoparasites were extracted from dissected the three different fishes collected from the Gomti River.

On a regular basis, fish various parts which are most susceptible to parasite infection such as the Gills, Liver, Gallbladder, and Gastrointestinal system are examined. After collecting the Helminth

parasites from the fish, we cleaned the helminth parasites in lukewarm/hot water. For morphological identification, the parasites were collected from infected areas for gross observation and identification. The external surface of the host body including scales, skin, and fins was examined by collecting mucus for ectoparasite. Gills were removed from the branchial cavity and placed on a glass slide for microscopic examination. Later, the morphological identification of the nematode, cestodes, and trematodes was done by using Light microscopy and Scanning Electron Microscopy imaging.

For the biochemical identification of parasites and hyperparasites standard biochemical tests as described in Bergey's Manual Determinative Bacteriology were used (e.g. oxidase, methyl red Nitrate, motility agar, test, etc.). Seasonal variations were analyzed to check seasonal variation in parasites and hyperparasites samples will be taken seasonally viz. Spring, summer, autumn, and winter.

Molecular characterization of hyperparasites was performed in which the genomic DNA was isolated by using a QIAamp'R DNA kit (QIAGEN, Hidden Germany). For phylogenetic studies, nuclear ribosomal and mitochondrial genes were amplified using PCR conditions described by little wood et al., (2008) and Kràlovà-Hromadovàet.al., (2010), respectively. Purified PCR products were sequenced using an applied Biosystem3130xl automatic DNA analyzer and Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA). The sequence was procured then BLAST by using NCBI website and further phylogenetic analysis was done by Clustal W, Clustal X, Mega, Bioedit Phylogeny etc. software.

A comparative analysis of parasitic prevalence is established which shows a steep rise in infection of freshwater fish *Channa punctatus* in the summer season than the other two sampled freshwater fish *Heteropneustes fossilis* and *Rita rita* indicative of a species specific host-parasitic

interaction based on the natural habitat of that organism and other physiological factors. This can be due to the fact that *C. punctatus* is bottom dwelling fish and is more prone to an infestation in terms of its habitat, survival rate, and sustainability.

In an another comparative analysis of parasitic prevalence, the trend line shows a weight-wise highest infection prevalence of helminth parasite in males than the females of *C. punctatus*, similar results were observed in *H. fossilis* wherein the mean percentage intensity was slightly higher in males than in females. In addition, while analysing the fish *R. rita*, the observations show a highest prevalence of helminths parasites in males than the females. The weight range during the course of study was maintained to be between 30 to 76 gm to keep up the uniformity of the experimental procedure, which more or less helped in concluding a sex specific parasitic infestation pattern.

While considering a comparative lengthwise infestation pattern of helminth parasite in freshwater fishes, it was surmised that the prevalence of helminths was highest (100%) in the lengthwise largest fish species of *C. punctatus,* when compared to the smallest fish (29.41 %). In *H. fossilis*, the highest infection rate of 41.66 % was observed in the largest fish, while the smaller fish showed the lowest infection range of 7.69 %. In *Rita rita*, the lengthwise highest infection rate of 66.66% in the largest fish was observed, while the smaller fish showed the lowest infection range of 24.24%. The range of length of fish was selected between 10 to 20 cm during the course of study to maintain the experimental standards, which helped in concluding a lengthwise parasitic infestation pattern in these three selected fish species, again indicative of the role of physiological factors like differential body type, size range, in deciding the susceptibility and sustainability of a host-parasite relationship.

The observations of parasitic prevalence, in the selected three freshwater fish, according to seasonal variation are indicative of season-

wise and species-specific variation in parasitic prevalence pattern, wherein the fish *C. punctatus* showed the highest infection range in both males and females in the summer and spring seasons. The other two fish species- *H. fossilis* and *R. rita* showed moderate infection range in the months of summer and spring. However, in the fish *R. rita* it has been shown that the parasitic prevalence increased in the months of winter also. This may be indicative of the nature dwelling and physiological response of these fish for survival in the winter season too in the wake of ecological niche. In all these fishes, the infection intensity remained moderate during the monsoons or rainy season due to the rise in water levels and changing habitat of fish along with the temperature variations. The observations in themselves are differential suggestive of a species-specific response of host-parasite interactions according to the climatic changes.

The present study showed that physicochemical parameters did not remain stable for a prolonged period at a particular place and show fluctuations from season to season. There was an increasing trend from winter to summer in water temperature in all the sites. The minimum and maximum temperatures recorded in different stations during different seasons ranged from 14 to 26°C. The pH value was the highest during summer and the lowest during autumn. The maximum pH value recorded was 8.46 during summer and the minimum was 6.4 during autumn.

The temperature and pH levels were the most important abiotic factor that affected the parasites at all life cycle stages along with the seasonal variation. The data obtained through these parameters indicate a positive correlation between water temperature and parasitic prevalence found in the fishes of the selected site of Gomti River. This goes same with the pH levels that too showed a positive correlation with all parasitic infections in collected fish samples of the Gomti River. Hence, it is established that these abiotic factors affect the parasitic prevalence accordingly and contribute broadly in the population dynamics studies of the parasite in fresh water fish.

Light microscopy and scanning electron microscopy were used to determine the morphology of the helminth parasites. These parasite specimens were identified as belonging to the *Senga* sp., *Pallisentis* sp., *Lycocestus* sp., and *Rostellascaris* sp.

Fish parasites, samples collected from different fishes, were pre-treated with physical and chemical methods before plating to eliminate common microbes. After these treatments, serial dilution method was performed for hyperperasites microbes bacterial cultures isolation. The gut of the fish is removed during sterile dissection. The parasites were placed in distilled water and crushed, extracted and kept into the first test tube that has saline before being serially diluted in the subsequent test tubes, in order to isolate bacterial hyperparasite. A micropipette is used to disperse the material from a few chosen test tubes onto petri plates containing medium, where it is then incubated for 24-48 hours at 37°C.

The samples were plated by serial dilution method on Nutrient Agar media and incubated at 37°C for 24-48 hours**.** Following that, the cultured medium is removed in order to identify the cultivated bacterial colonies and characterize the colony shape. These colonies were selected on the basis of morphological features and further purified and subjected to screening.

A total of 5 samples were collected from different infected fish. The bacterial colony was distinguished based on its morphological characteristics. Colony morphology can help determine the kind of bacteria present. According to their shape, margin, and elevation, bacterial colonies are categorised. Bacterial colonies are classified as circular, irregular, filamentous, and rhizoid based on their shape. They can be categorised as raised, convex, flat, umbonate, and crateriform according on their elevation. Colonies are categorized as a whole, undulate, filiform, lobate, and curled according on how their margins are shaped. These bacteria collected from different infected fish were typically in irregular circular or

filamentous forms. Based on the elevation, these bacteria were raised, convex, flat, and umbonate type. On the basis of margin, they were categorized into entire, lobate, and undulate types.

The five bacterial strains HP-, HP-2, HP-3, HP-4, and HP-5 were further selected for Bergey's manual of systematic bacteriology, which is the main source for defining the characteristics of prokaryotic organisms, highlighting bacterial species, using each characterizing feature. For identification, different physiological and biochemical tests were accomplished as explained by Williams *et al.* (1989) and Bergey's manual **(**Holt *et al.*, 1994). All the bacterial strains HP-, HP-2, HP-3, HP-4, and HP-5 strains were characterized by streaking procedures on the nutrient agar plates and incubated for 24-48 hours at 37˚C for the specified characteristics.

Gram staining is a general method used to discriminate bacteria based on their dissimilar cell wall components. The Gram staining technique differentiates between Gram-positive and Gram-negative bacteria individuals by coloring these cells red or purple. Bacteria isolated from the samples HP-1 to HP-3 were found as Gram-positive bacteria in purple color**.** HP-4 and HP-5 were found as Gram-negative bacteria.

Fermentation test is used to define whether or not bacteria can exploit a specific carbohydrate. The sample numbers HP-2, HP-3 and HP-5 showed a positive result for glucose sugar. The sample numbers HP-1, and HP-4 isolates showed a negative result for the sugar fermentation

The goal is to determine if the microorganism can use lactose, a sugar, as a carbon source for fermentation. Analysis for lactose fermentation test suggested that a positive test for samples HP-1, HP-3, and HP-4, whereas samples 2, HP-2 and HP-5 were negative.

The goal is to determine if the bacterium can use mannitol, a carbohydrate (sugar), as a carbon source for fermentation. The pH of the medium will fall if mannitol is fermented to yield acid end products. The presence of acid is indicated by a pH indicator in the medium turning colour. Analysis for the VP test suggested that a negative test for samples HP-1, HP-3, and HP-5, whereas samples 2, HP-2 and HP-4 were positive.

To check out whether a microorganism makes acetylmethyl carbinol from glucose fermentation, do the Voges-Proskauer (VP) test. A pink-red tint that appears at the surface after 15 minutes or longer following the addition of the reagents, signifying the presence of diacetyl, the acetoin oxidation product, is indicative of a positive VP test. Analysis for the VP test suggested that a negative test for samples HP-1, HP-2, and HP-4, whereas samples 2, HP-3, and HP-5 were positive.

Methyl red is a pH indicator to decide whether the bacterium brings out mixed acid fermentation. Some bacteria have the capacity to use glucose and transform it into an end product that is a stable acid, such as lactic acid, acetic acid, or formic acid. The test microorganisms are cultivated in a broth medium containing glucose for the methyl red test. The colour of the methyl red changes from yellow to red when introduced to the broth culture if the bacteria can use glucose and produce a stable acid. Analysis for the methyl red test suggested that a positive test for samples HP-1, HP-2, and HP-4, whereas samples 2, HP-3 and HP-5 were negative.

The indole test illustrates how some bacteria may break down the medium-accumulating amino acid tryptophan into indole. The test for indole synthesis is crucial for identifying enterobacteria. Analysis for the methyl red test suggested that a positive test for samples HP-1, HP-2, HP-3, and HP-4, whereas sample HP-5 was negative.

The Catalase test is used to identify the occurrence of the enzyme catalase, which changes hydrogen peroxide into H_2O and oxygen. When a colony of the microorganism interacted with hydrogen peroxide, then bubbles formed specifying a positive response, whereas a lack of bubbles shows a negative reaction (Chester, 1979). Analysis for the catalase test suggested that a negative test for samples HP-1, HP-2, HP-3 and HP-4, whereas sample HP-5 was negative.

The important facts required for correctly identifying the genera of distinct bacteria inside a sample could be revealed through biochemical reactions. Bacteria naturally create enormous amounts of enzymes, and it is owing to these enzymes that they may be identified using biochemical techniques. Since bacteria have unique enzymatic profiles, it is typically possible to identify a bacterium by the type of enzymes that it produces.

According to the 16S rRNA sequence studies, HP-1, HP-2 and HP-3 isolates were found to be *Bacillus firmus, Brevibacillus agri* and *Paenibacillus cisolokensis*, respectively. Whereas on the basis of the 16S rRNA sequence studies, HP-4 and HP-5 isolates were found to be *Aeromonas veronii* and *Plesiomonas shigelloides*, respectively. The sequences obtained were compared with the NCBI gene bank database using the BLAST search program (http;//www.ncbi.nlm.nih.gov). Further, the sequences of the bacterial isolates were used for the construction of the phylogenetic dendrogram to know the genetic relatedness between the bacterial isolates. The phylogenetic analyses were done by their neighbour obtained from Genebank.

The present study was undertaken to study the incidence of helminth parasites in fishes with special reference to water quality parameters and to correlate the parasitic prevalence and various physicochemical parameters. Infection patterns of reported parasites- *Senga* sp., *Pallisentis* sp., *Lycocestus* sp., and *Rostellascaris* sp., were greatly influenced by season, fish species, and type of water body. The

study showed that some of the physicochemical features showed a significant positive correlation with the prevalence.

The purpose of the thesis project was to find hyperparasite microorganisms in the guts of various fish, which lead to a brief framework of the bacteria present and protect fish. Three different fish species from the Gomti River located at Lucknow were selected for the presented work of analysis specifically and a comprehensive study has been accompanied. These fish were taken at the Ghaila site of Lucknow. Due to pollution in the river, the chances of several different types of micro-organisms especially bacterial species in the fish gut were more possible and anticipated. Then, different fish were taken for an examination with all the necessary information needed for a detailed study. Fish guts or intestines were dissected in a sterile environment for the collection of hyperparasite bacteria. These hyperparasite bacteria were grown in the nutrient agar media. After a series of dilutions, bacteria were seen to be growing on the nutrient agar plate, after performing properly the process of spreading, streaking, and incubating them at the given accurate temperature.

In general, infection patterns of parasites in fish populations are influenced by the availability of infective larvae, feeding habits of the host, age and sex of the host, mortality of parasites, and abiotic factors such as salinity, temperature, and season of the year. The relationships between prevalence and mean intensity of infection of the parasites have been well addressed in the present study with special reference to the sex and size of host fish, and seasonal variations during sampling durations.

In this study, the diversity and seasonal variations of helminthic parasites in snakeheads are investigated. These studies will improve our knowledge of host-parasite interactions, allowing us to more effectively combat infectious diseases and increase fish production. Throughout the course of the study, the experimental fish were separated into several length groups. The prevalence was observed to be higher in the group of

fish that were longer than 18 cm, with a frequency of 100%. A greater mean intensity of 2.47 was observed in the group of medium-sized fish.

Finally, it can be summarized that season, sex, and breeding period as factors influencing parasite occurrence and abundance. Fish weight and gonads are also important driving factors when correlated to parasite infection. When considering the relationships between fish reproduction and parasites, some cestodes with a complex life cycle show seasonal patterns of infection compatible with host reproduction without a host immune response that could suggest evolutionary host-parasite interactions. Such a pattern may be induced by increasing levels of host sex hormones during fish spawning, which leads to the hypothesis that a high level of hyperparasitism co-occurs with fish reproduction.

Fish represent an important biological group that is significant in determining the sustainability of the aquatic ecosystem balance. Parasites are a major threat to their sustainability and any change in the habitat will be an alarming situation. Hence, keeping in view the negativity of parasitic influence, it is a need to study the population dynamics of both the parasites and hyperparasites which will be helpful in implementing strategies in their bioremediation.