Purification and Characterization of *Pomphorhynchus kashmirensis* Somatic Antigens

DISSERTATION

Submitted in partial fulfillment of the requirements for the Award of the Degree of

MASTER OF PHILOSOPHY

In

ZOOLOGY (PARASITOLOGY)

By

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October, 2011

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CERTIFICATE

This is to certify that the Dissertation entitled **"Purification and Characterization of *Pomphorhynchus kashmirensis* Somatic Antigens."** submitted to the University of Kashmir for the award of the Degree of *MASTER OF PHILOSOPHY IN ZOOLOGY*, is the original research work of ***Ms. Sumayya Nazir,*** a bonafide M. Phil. Research Scholar of the Department, carried out under our supervision. The dissertation has not been submitted to this University or to some other University so far and is submitted for the first time. It is further certified that this dissertation is fit for submission for the degree of Masters of Philosophy (M. Phil.) in Zoology and the candidate has fulfilled all the statutory requirements for the completion of the M. Phil. Programme.

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DEDICATED TO

MY FAMILY

AND

WELLWISHERS

ACKNOWLEDGMENT

Thanks to Almighty Allah, the Creator of the universe, Who poured in me the spirit and enthusiasm of understanding research in versatile fields like molecular in particular, which indeed is a beginning in the said field here. To achieve the goals in the present endeavor were not less than a challenging task for me.

It gives me immense pleasure to express my sincere regards and deep sense of gratitude to my supervisor, Dr. Md. Niamat Ali, Sr. Assistant Professor, PG Department of Zoology, University of Kashmir, for his valuable suggestions and constructive criticism that has been crucial in the completion of this work. I am especially grateful to him for proof reading with utmost care and patience.

I owe lots of debts to my co supervisor Prof. M. Z. Chishti, Professor Emeritus, CORD for his fatherly affection, expert views and valuable suggestions that indeed helped me to vacate any room for any mistakes and shortcomings.

I also express my gratitude to Heads of the Department for their kind cooperation and providing me the laboratory facilities without which the work would not have been completed.

I would like to acknowledge the support provided by all other teaching staff members of the P.G. Department of Zoology, University of Kashmir for their constant encouragement and valuable suggestions. Sincere thanks to non teaching staff of the department also who helped me in one or the other way during my studies.

I am greatly thankful to my seniors especially Mr. Bashir Ahmad & Dr. Riyaz Ahmad Mir who stood by me during this period. It is my duty to express my million dollar thanks to them for their unsitting support and encouragement.

My heart gets overwhelmed with joy while expressing my special thanks to my grandparents and parents for their moral support and wishing best for me in their precious duas which indeed is immensely required till the last breath of my life. It is worthy to mention the name of my younger brother Abid who spent precious hours with me while typing. I also wish to express my appreciation to my nephews especially Mohammed Sameem, Faraz, Ibrahim and Usman who stood by me though they missed my love and affection during the research tenure.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHS

ABSTRACT

Fish fauna is ecologically very important and plays a vital role in food chain. There is a great scope for the development of fishery resources in the valley of Kashmir so as to overcome the prevailing animal protein defiency in the diet of the local people. In country like India, intake of meat & milk is low, so fish has special importance as a supplement to ill- balanced cereal diet. Fish flesh is also a highly perishable commodity constituted by 60-80 %water & 13-20 % protein & being low in cholesterol & free from fats. Fish protein hydrosylates also have antioxidant properties. The flesh also contains phosphorus & vitamins. Freshwater fishes form one the important food sources in both the developed as well as underdeveloped countries.

A lot of research work has been done on various aspects of these vertebrates but a meagre work is available on the molecular aspects particularly immunogenicity of parasites of the fish host. Therefore, study on the characteristics of the protein profile of the fish parasite *Pomphorhynchus kashmirensis* was undertaken. The fishes were collected from Dal Lake and River Jhelum and it forms the first study of its type in this part of the country.

In order to have a glimpse of the related work done in the past, an attempt was made to review the available literature on the subject. Parkhouse, *et al.,* (1987) characterized and studied the protective effect of nematode antigens. Bunyatova and Elchiev (1989) constructed electrophoretic spectrum of proteins of an acanthocephalan *Leptorhynchoides plagicephalus.* Coscia and Oreste (2000) investigated the presence of antibodies against protein antigens of the nematode parasite *Pseudoterranova decipiens* in the plasma and bile of the Antartic Teleost, *Trematomus bernachii.* Knopf, *et al*., (2000) studied the humoral response of the European eel Anguilla *Anguilla* elicited by an experimental infection with a swim bladder nematode *Anguillicola crassus*. Noga, *et al.,* (2000) purified antimicrobial proteins from rainbow trouts and sunshine bass by RP-HPLC and SDS-PAGE. Saifullah, *et al.,* (2000) worked on the excretory /secretory (ES) metabolic products released by *Gastrothylax crumonifer* (trematode:Digenea) during in vitro incubations and the somatic extract of the adult parasites were analyzed using PAGE. As obvious, it was not possible to review all the available literature on the said topic, the important ones were penned down in the dissertation.

Studies on the fishes has showed that *Pomphorhynchus kashmirensis* is usually found in the intestine by boring its proboscis into it and thereby produces lesions and makes it prone to further secondary infections. Besides, it is also present

in the body cavity, liver and spleen of its fish host. It causes tremendous damage to the intestinal walls at the site of its attachment. The lamina propia gets thickened and goblet cells in this region become more prominent and their number also gets increased (Yildiz, *et al*, 2004; Ahanger, *et al.*, 2008).This parasite is considered to be one of the most dangerous parasite responsible for fish mortality and morbidity (Ahanger, *et al*., 2008). Hence the need was felt to study this parasite at molecular level so that our fish fauna are spared from them.

A total of 363 fish specimens of *Schizothorax* species were collected and out of which, 203 fishes were collected and examined from Dal Lake and 160 fishes were collected and examined from River Jhelum during the present study. The host was collected with the help of local fishermen in live condition. Fishes were dissected & body cavity was thoroughly examined for any parasite. Intestines were placed in Petri dish containing normal saline (0.75%Nacl, Cable 1958) to allow adhering parasites to be released from the lumen. *Pomphorhynchus* was carefully removed from the intestines with the help of brush and needle. A regular record of this parasites was recorded and then subjected to various immunological and biochemical techniques in order to understand the nature of somatic antigens.

Out of 203 specimens examined from the Dal Lake only 42 specimens were found infected with the *Pomphorhynchus kashmirensis* which constitutes the prevalence of 20.68%. Similarly out of 160 specimens examined from the River Jhelum only 52 specimens were infected with the *Pomphorhynchus kashmirensis* which constitutes 32.5% prevalence. Also, *Pomphorhynchus kashmirensis* showed a wide host range and was successfully establishing in various species of Schizothorax. The highest prevalence were found in *S. niger* (30 %) (26.19% in Dal and 34.85% in Jhelum) followed by *S. curvifrons* (27.11 %) (19.11% in Dal and 38% in Jhelum) and least prevalence were found in *S. esocinus* (17.89%) (13.72% in Dal and 22.73% in Jhelum).

Pomphorhynchus infection also revealed definite seasonal prevalence of infection in all the three species of *Schizothorax,* with highest infection in summer and lowest in winter. There was a gradual increase in the prevalence rate from spring to summer and falls down with onset of autumn and least observed prevalence during winter season. In summer the prevalence was 34.54% (*S. niger* 46.34%, *S. curvifrons* 31.11% and *S. esocinus* 30%) and the least prevalence was found during the winter season 8.16% (*S. niger* 14.28 %, *S. curvifrons* 6.89 % and *S. esocinus* 9.52%).

Gender wise observations were also made which revealed that the sex wise differences were not much prominent but in most cases males 30.30% (*S. niger* 32.50%, *S. curvifrons* 27.65% and *S. esocinus* 28.94%) were found to be more infected than females 27.27% (*S. niger* 27.14%, *S. curvifrons* 36.61% and *S. esocinus* 15.78%).

To characterize the somatic antigens from *Pomphorhynchus kashmirensis*, the sera of the fish was used as a source of antibodies. For this purpose affinity chromatography is the most appropriate technique to be utilized. From this technique the eluted bound protein was dialyzed extensively against 20 mM Tris-saline buffer, pH 7.4, concentrated with PEG 20,000 and was designated as affinity purified *P. kashmirensis* somatic antigen (Aff-PSAg). The protein content of the antigen was determined spectrophotometrically.

SDS-PAGE confirmation of purified antigen Aff-PSAg was done by SDS-PAGE followed by staining with Coomassie Brilliant blue. Electrophoretic separation of Aff-PSAg resolved into 5 prominent polypeptides of molecular weight ranging from 29 to 66 kDa which is inferred to the presence of 5 or more number of active somatic antigens of *P. kashmirensis*. To measure the antigenicity of the purified antigens the commonly used serotest viz., Ouchterlony double diffusion (ODD) was used. Crude somatic and partially purified pooled fractions of *Pomphorhynchus kashmirensis* were subjected to double immunodiffusion against rabbit hyper immune sera. Ouchterlony gel diffusion test of somatic antigens showed one precipitation arch against heterogeneous hyper immune sera and many precipitation arches against homogenous hyper immune sera.

In nutshell, it is observed that the somatic antigens derived from the *Pomphorhynchus kashmirensis* can be used as good immunogens and hence can be exploited for mounting the protective immune response in fish. The results of the present study suggest that low molecular weight antigens of *Pomphorhynchus kashmirensis* deserve further investigation.

CHAPTER: 1

INTRODUCTION

The Jammu and Kashmir is the northernmost State of India. It is situated mostly in the Himalayan Mountains. Jammu and Kashmir shares a border with the states of Himachal Pradesh and Punjab to the south and internationally with the People's Republic of China to the north and east and the Pakistani administered territories of Azad Kashmir and Gilgit-Baltistan, to the west and northwest respectively.

Jammu and Kashmir consists of three regions: Jammu, the Kashmir valley and Ladakh. Srinagar is the summer capital, and Jammu, its winter capital. While the Kashmir valley, often known as *Paradise on Earth*, is famous for its beautiful mountainous landscape. Jammu and Kashmir is home to several valleys such as the Kashmir Valley, Tawi Valley, Chenab Valley, Poonch Valley, Sind Valley and Lidder Valley. The main Kashmir valley is 100 km (62 mi) wide and 15,520.3 km^2 (5,992.4 sq mi) in area. The Himalayas divide the Kashmir valley from Ladakh while the Pir Panjal range, which encloses the valley from the west and the south, separates it from the Great Plains of northern India. Along the north-eastern flank of the Valley runs the main range of the Himalayas. This densely settled and beautiful valley has an average height of 1,850 metres (6,070 ft) above sea-level but the surrounding Pir Panjal range has an average elevation of 5,000 metres (16,000 ft).

Nature has been very kind & benevolent to the people of Kashmir. Nature has bestowed innumerable gifts to this piece of earth & thus the beauty of the valley has

no comparison in the world. The freshwater habitats of different types that add splendour to the unsurpassed beauty of the valley also form unrivalled highland fishery resources characteristics of the Himalayas.

Almost all water bodies are a source of tourist attraction & also provide a substantial quantity of fish & also support some cottage industries. Amongst these habitats the Lentic habitats are represented by tarns, lakes, ponds, wetlands and roadside ditches and similar other small aquatic ecosystems. The lotic habitats are represented by the River Jhelum and numerous cold water hill streams which directly or indirectly join the River Jhelum.

Study sites

Dal Lake is situated in Srinagar, the summer capital of the northernmost Indian state of Jammu and Kashmir. The urban lake, which is the second largest in the state, is integral to tourism and recreation in Kashmir and is nicknamed as the "Jewel in the crown of Kashmir" or "Srinagar's Jewel". The lake is also an important source for commercial operations in fishing.

Fish fauna of the lake include *Cyprinus carpio specularis* (economically important), *C. carpio communis*, *Schizothorax niger*, *S. esocinus*, *S. curviformis* and *Crossochelius latius*. It is also reported that *Cyprinus*, introduced during early sixties, is dominant and that the indigenous species *Schizothorax* is showing a declining trend.

The RiverJhelum (Vyeth in Kashmiri, Vetesta in Sanskrit and Hydaspes in Greek) is the main waterway of the valley of Kashmir. It initiates from a beautiful spring called Verinag. This spring is situated at the foot of a spur of the Pir Panjal Mountain.

The major fish fauna of river Jhelum comprises of exotic carp, (*Cyprinus carpio)* and indigenous carp (*schizothorax* spp). *Cyprinus carp* is represented by *Cyprinus carpio specularis*, and *C. carp communis*, while *schizothorax* sp is represented by *S. esosinus, S. curvifrons, S, planifrons, S. labiatus, S. punctatus, S. micropogon, S. niger* and one specioes of *Oreinus-O.(Schizothorax) plagiostomus.* The other fishes though rarely found are L*abeo dero, L. dyocheilus, Crossocheilus latius, punctius Conchonius* among Cyprinidae; *Glyptothorax kashmirensis, Gyptosternum reticulatum(sisorridae)* and Botia birdi, *Nemachilus kashmirensis, N. rupicola and N. marmoratus* among *Cobitidae.*

The vastness of the Dal Lake, which stands next to Wular Lake and the River Jhelum strongly, indicates the richness of the fishery. There is a great scope for the

development of fishery resources in the valley of Kashmir so as to overcome the prevailing animal protein defiency in the diet of the local people. In country like India, intake of meat & milk is low, so fish has special importance as a supplement to ill- balanced cereal diet. Fish flesh is also a highly perishable commodity constituted by 60-80 %water & 13-20 % protein & being low in cholesterol & free from fats. Fish protein hydrosylates also have antioxidant properties (Samaranayaka, 2010). The flesh also contains phosphorus & vitamins. Freshwater fishes form one the important food sources in both the developed as well as underdeveloped countries.

Parasitism

The word parasite is derived from the Greek words Para (meaning beside) and sitos (meaning food). Parasites can be described as living organisms that are associated with food for all or part of their life cycle. The organism providing the food is generally called as the host and the organism deriving its nourishment from the host is called as the parasite. The host parasite relationship is a heterogenetic association between the two organisms in which one generally small called as parasite is metabolically dependent upon the other organism called as the host. Fishes which include the Agnatha, Dipnoi, Chondichthyes, Osthicthyes, etc represent a diverse group including over 20,000 species occurring in a variety of environments and acting as the hosts for many parasites. The aquatic environment of freshwater encompasses a wide variety of features, virtually all of which influence the maintenance of homeostasis, essential for growth and reproduction of fish. If altered beyond acceptable limits, they may predispose to or may cause a wide range of diseases. Diseases in fish are thus closely related to environmental stress.

The pollution of Dal Lake and the river Jhelum by various agents & sources has serious adverse effects on fishes. Pollution destroys the reproductive conditions & it also disrupts the metabolism of fishes & leads to the mortality of the fish food organisms. This pollution makes them prone to various kinds of infectious diseases especially to the parasites (Nacher and Sures, 2007; Fotedar and Qadri, 1974). These parasites are always in complex dynamic equilibrium with the free living communities of plants & animals. Fishes are the apex of the predator –prey pyramid within fresh waters & therefore, tend to be infected by a considerable range of parasites, which may occur in large no's. This is the normal condition found in any natural environment.

All living beings in certain circumstances become subjected to diseases & fishes make no exception. Fishes like other animals fall prey to large no. of diseases,

caused by a variety of parasites. Many such parasites are responsible for high mortality rates of fishes, particularly the younger stages. Among various parasitic diseases of fishes-helminth parasites form the main group.

Helminth parasites are the most common & important among various fish parasitic diseases affecting the fish in different ways. Fishes fall prey to the infection of parasites in their larval stages, e.g. choking of gills of fingerlings is caused by monogenetic trematode which affects their respiratory activity causing their mortality. Acute inflammation of intestinal tract is caused by even few acanthocephalids which in turn is capable of influencing the growth rates of fishes. Infection of fish population by Helminth parasites slows growth, increases susceptibility to secondary infections & thus increases their predation. Parasites also influence sex hormone levels & stress hormones (Nacher and Sures, 2007).

Not only the fishes are affected by the parasites but man also comes under their effects. Laryngopharyngitis is a well known disease which in man is caused by improperly cooked fish infected with *Clinostomum marginalis* & *C. complanatum.* A potential life threatening allergic like reaction in man is caused by *Anisakis simplex.* Similarly acute urticaria in man is caused by fish infected with *Anisakis simplex* (Ventura, *et al.*, 2008). In short, humans are exposed to these parasites at the end of the food chain. The diseases are characterized by gastrointestinal, neurological & cardiovascular disturbances, which in severe conditions may even lead to the death of humans.

Keeping these aspects in view it is evident that helminths cause a great damage to the fishes. If this damage is to be reduced then steps need to be taken to control these parasites & the foremost work is to generate a baseline data about the helminths infecting the fish fauna. A lot of research work has been done on the Helminth parasites regarding their morphology, physiology, reproduction, nutrition, taxonomy, anatomy, systemic position, their serological & haematological effects on hosts & so on but little work has been done on their molecular aspects in order to detect their antigenic properties which can be utilized for the benefits of fish host e.g. fish vaccines against helminths.

The present study mainly deals with the *Pomphorhynchus kashmirensis* which is commonly found in the gut of infected *Schizothorax sps* inhibiting the waters of the beautiful Dal- Lake and the River Jhelum. *Pomphorhynchus kashmirensis* is usually found in the intestine by boring its proboscis into it and thereby produces lesions and makes it prone to further secondary infections. Besides, it is also present in the body

cavity, liver and spleen of its fish host. It causes tremendous damage to the intestinal walls at the site of its attachment. The lamina propia gets thickened and goblet cells in this region become more prominent and their number also gets increased (Yildiz, *et al.,* 2004).This parasite is considered to be one of the most dangerous parasite responsible for fish mortality and morbidity (Ahanger, *et al*., 2008).

Immunological response of fish to *Pomphorhynchus kashmirensis:*

On entering into its host *Pomphorhynchus kashmirensis* is continuously being challenged by the immune system of the host and the host immune system in turn generates specialized class of proteins called antibodies to eliminate the parasite. These antibodies are highly specific and are directed against the surface/somatic or secretory proteins of the parasite. There is little doubt that the host immune response represents the major defence against the parasitic attack (Wakelin, 1996). Upon entering the host, parasites are exposed to the effectors of the immune system, which may result in the clearance of the infection. In response of the threat imposed by the immune response, parasites have evolved a variety of strategies aimed at manipulating host immunity (Damian, 1997; Schmid-Hempel, 2008). These strategies of immune evasion are supposed to favour the establishment, the growth and the reproduction of the parasites within the host. Helminth parasites are masters in their ability to depress the host immune function. Thus, the immunological system has not been so successful in producing resistance to the parasites. The immune system is relatively inefficient in controlling the helminth parasites. After all these organisms have adapted to an obligatory parasitic existence. Parasitic helminths are, therefore not maladapted pathogenic organisms but fully adapted obligate parasites whose very survival depends on reaching some form of accommodation with the host.

Diagnoses and control measures of *Pomphorhynchus kashmirensis:*

The basic aim of any study related to parasitological and immunological problems is to contribute directly or indirectly towards the control of infection. An efficient control of the disease depends on the correct diagnosis, correct identification of causative agent and then the integrated application of several control measures as shown in Fig. 1.

Besides, several serological and haematological studies have been extensively used for the diagnosis of the fish diseases but little work regarding the molecular

aspects of the parasites have been carried out. However, a lot of research work on such properties of the parasites have been carried out in abroad on various parasites using the different techniques- chromatography, SDS-PAGE, etc because of their convenience (Parkhouse, *et al*., 1987; Kennedy, *et al.*, 1989; Woo and Thomas, *et al.*, 1991; Joshi and Singh, 1999; Knopf, *et al.*, 2000; Saifullah, *et al*., 2000; Hamwood *et al.*, 2000).

The control measures of *Pomphorhynchus kashmirensis* can be achieved by following a proper management techniques and using the antehelminthics. However the management programmes have their own limitations and also the parasites generally show resistance towards the antehelminthics. The massive and indiscriminate use of the drugs in many parts of the world has been responsible for the appearance of resistant isolates of parasites to most of the chemical groups employed. Thus, indicating the necessity for studies on alternative effective measures for control of these parasites. Innate resistance to this parasite and vaccination are the two effective methods for its control.

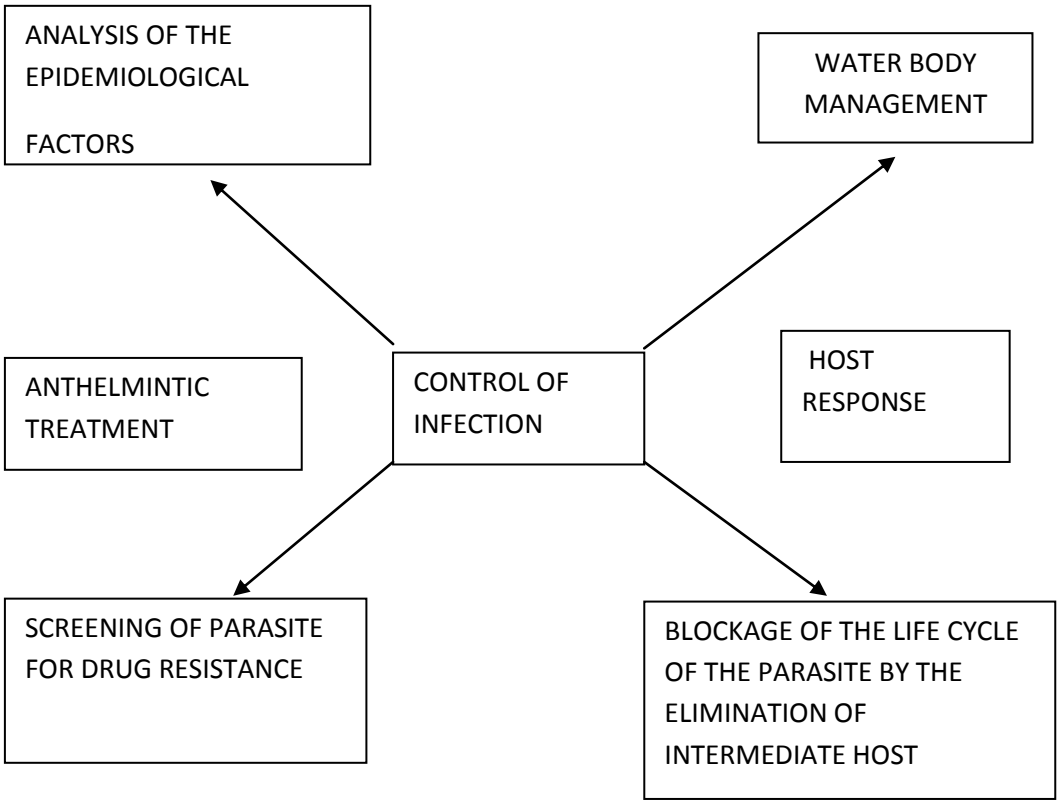


Fig. 1: Strategies of parasite control.

The present endeavour mainly deals with Epidemiological and immunological studies especially the nature of surface proteins/somatic antigens of the *Pomphorhynchus kashmirensis* using chromatography and SDS-PAGE and confirming the antigenicity strength by Ouchterlony double diffusion test (ODD) which will thus provide the baseline data about the antigenic properties of the *Pomphorhynchus kashmirensis* and hence can be utilized for the preparation of vaccines against it. The introduction of such a vaccine in the fish will boost its adaptive immune response and thus in turn, will be strongly in a position to combat the infection of *Pomphorhynchus kashmirensis* much before its entry in it.

Description of the *Pomphorhynchus kashmirensis;*

Body is distinctly divided into proboscis, neck, and trunk. Proboscis is cylindrical and beset with hooks in 14 longitudinal rows with 11-12 hooks in each row. The hooks vary in size from 0.007-0.033 and 0.09- 0.36mm and testis length is 0.32 x 0.23mm. Neck is long and bulla at its anterior end is quite distinct. Neck lodges proboscis receptacle which extends to the base of the neck. Trunk trappers posteriorly with terminal end blunt.

MALE: smaller than female it measures 5.25mm x.75 mm in size. Two oval testes lie tandem in the middle trunk region. Cement glands are six in number; their arrangement being (1+3+2); saefftigen's pouch is quite distinct and opens into bursa.

FEMALE: it measures 6.35mm in length. Ovary broken into large number of ovarian balls and is covered over by a ligament sac. Uterine ball is funnel shaped and continuous with the posterior extremity of the ligament sac. Uterine tube passes into a long muscular uterus. Terminal end of the genital tract is non-muscular representing vagina.

Life cycle: Acanthocephalans (*Pomphorhynchus kashmirensis**)*** are widely distributed intestinal worms parasitizing fish fauna (Taraschewski, 2000). Adult helminths live inside the intestines of the final host and absorb the nutrients across, as they lack the alimentary canal. The life cycle of acanthocephalans involves a vertebrate definitive host and an arthropod intermediate host which may be an amphipod or copepod. Embryonated eggs of female acanthocephalan reaches the water with the gut contents of the host, where an intermediate host eats the eggs. These eggs contain the first stage larvae called acanthor. The acanthor hatches inside the intestine of intermediate host into a cystacanth. A fish gets infected by feeding on infected intermediate host having an infective stage cystacanth.

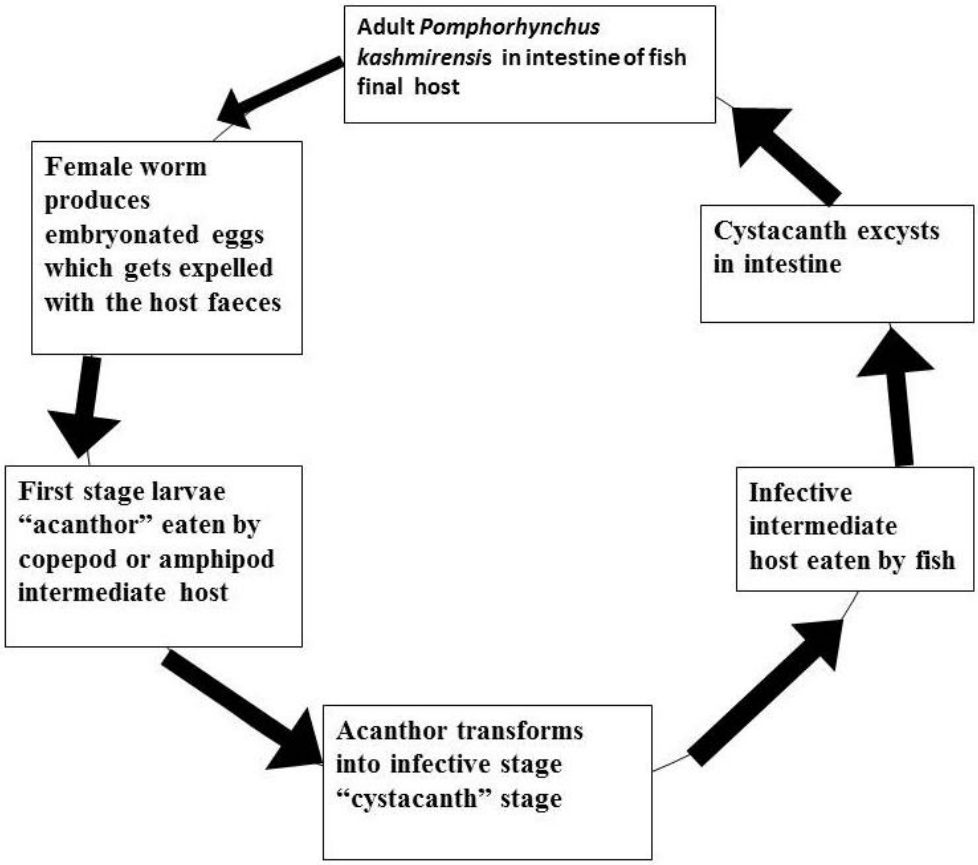


Fig.2: Life cycle of *Pomphorhynchus kashmirensis*

CHAPTER: 2

REVIEW OF LITERATURE

The present work dealing with the isolation and characterization of parasitic antigens has been studied across the globe right from early nineteenth century. In order to have an idea about the previous work carried on the aspect it was necessary to have a clear idea about the previous work so an attempt was made to review the available printed literature on the subject. As it was not possible to review all the work done so far, therefore, the important ones of last decades are reviewed in the following pages. In order to have a clear understanding the literature has been revised under two headings.

2.1) Prevalence of *Pomphorhynchus kashmirensis*

2.2) Immunology and biochemistry of parasites

2.1) Prevalence of *Pomphorhynchus kashmirensis*

Pomphorhynchus kashmirensis is very much prevalent in the fishes of the Kashmir Valley especially in *Schizothorax* species that is evident from the literature cited below.

Datta (1936) described helminth parasites with special reference to Acanthocephalans of fishes. Kaw (1941) provided a detailed account of helminth fauna of fishes in Kashmir.

Cushing (1942) described the various abiotic factors responsible for the parasitic infection in fishes. The role of temperature has been greatly emphasized by the author for the incidence of infection and hence the antibody production. Bisset (1948) worked on the immune system of fishes and concluded that infection increases with a steep rise in temperature.

Fotedar and Qadri (1974) studied the impact of introduced carp and the deteriorating ecological conditions of lakes of Kashmir especially Wular and Dal on *Schizothorax* and *Orienus*. Amin (1975) studied the host and seasonal associations of *Acanthocephalus parjsidei (*Acanthocephala: Echinorhynchidae) in Wisconsin fishes and observed that highest abundance and maturation occur during summer and recruitment during summer and autumn.

Andryuk (1979) investigated the life cycle patterns of acanthocephalans with special reference to *Acanthocephalus lucii.*

Radujkovic, *et a*l., (1983) investigated the ill effects of some parasites especially Acanthocephla and Nematoda on the fish host, *Chelon labrosus.* Acanthocephalan alone was found to be responsible for macrocytic anaemia while as nematodes were neutral in this regard. They further explained that acanthocephalans may also responsible for Vitamin B and folic acid deficiency in the fish host.

Gleason (1984) studied the seasonal prevalence, intensity of infection, infrapopulation and dispersal pattern of *Pomphorhynchus bulbicolli* in *Hyperntelium nigricans*. Significant differences were found in the seasonal prevalence and mean intensity of infection; both were low in winter and high in summer.

Jr. Williams and Rogers (1984) identified new species of *Pomphorhynchus* which was collected from 14 host species representing 7 families and six orders of fishes from northern Florida and southern Alabama. It differed from all known species of *Pomphorhynchus* by possessing 20-23 proboscis hooks per row. It differed from *P. rocci* by having a longer neck in relation to trunk length, larger hooks, a longer proboscis, and a smaller trunk.

Amin (1987) worked on *Pomphorhynchus bulbocoli* from fishes of Wisconsin lake and concluded that its shows seasonal prevalence and that host specificity were not so specific.

Brown (1989) while working on seasonal dynamics of the *Pomphorhynchus laevis* in its intermediate and preferred definitive hosts observed that the rate of parasitic growth increased with water temperature.

Jha, *et al.,* (1992) reported seasonal occurrence of helminth parasites in fishes, and showed moderate to high occurrence during different months, while digenetic trematodes were having quite low prevalence and even the infestation was absent during certain months of investigation period.

Molloy, *et al.,* (1995) studied the population biology of Pomphorhynchus laevis in brown trout (*Salmo trutta*) caught from two lakes in the Burrishoole River system in Irish Republic.

Majidah and Khan (1996) studied the population dynamics of nine species of helminth fauna including *Diplozoan kashmirensis, Clinostomum schizothoraxi, Camallanus fotedari, R. himalayai, R. kashmirensis, Adenoscolex oreini, Gangesia fotedar, Pomphorhynchus kashmirensis, N. manasbalensis* from seven species of fish (*Schizothorax hugeli, S.esocinus, S curvifrons, S niger, Oreinus plagiostomus, Nemachilus kashmirensis, C. carpio specularis* of Wular lake, Kashmir, India.

Morand (1996) investigated that parasite body size is positively correlated with the host body size. He further proved that parasite body size is related to host longevity. Long lived hosts would provide more energy and would harbor more long lived parasites and hence the larger ones.

Yousuf and Pandit (1996) studied the developmental process of the *Schizothorax niger* heckle inhabiting different lakes of Kashmir Valley. They observed a steep rise in temperature hastens the hatching which usually takes place after 13 days of fertilization in a temperature range of 9-12^{O}C. Its breeding grounds are usually located in the shallow parts of lakes on spring beds. Bakker, *et al.,* (1997) for the first time showed that both the parasite colour and changed intermediate host behavior promote the transmission of *Pomphorhynchus laevis* to its next host.

Chishti and Peerzada (1998) carried out a helminthological survey on the fishes of Wular. Out of 1662 specimens 155 were infected with acanthocephalans and out of which maximum infection was seen with Pomphorhynchus kashmirensis and least with *N. manasbalensis*. Maximum infection was recorded in spring season in all hosts and was usually dominated by males. An increase in the mean number of parasites per host with an increase in the host length was also evident.

Grutter (1998) observed the prevalence and number of *Benedenia* species on *Hemigymnus melapterus and was* significantly greater on fish from the reef flat than from the reef slope at Heron Island. This difference in parasite abundance between the habitats suggests that *H. melapterus* does not move between the reef flat and reef slope separated by only a few hundreds of meters.

Khan and Majidah (1999) studied the impact of physiochemical parameters on the diversity of fish parasites and concluded that parasite infection showed a regular seasonal trend. Infection was highest in the late summer and early autumn months. Infection was also influenced by other biotic and abiotic factors also.

Kennedy (1999) investigated the possibility of post-cyclic transmission in *Pomphorhynchus laevis.* Rainbow trout were exposed to *P. laevis* in naturally infected *Cottus gobia, Nemacheilus barbatulus, P. phoximus* and *L. cephalus*. Post cyclic transmission of gravid parasites could occur from *C. gobia* but not from *L. cephalus* which indicates that this failure to transmit larger parasites of either sex reflects the age and so development of the proboscis bulb of *P. laevis* and the extent of the host encapsulation response.

Ahmad and Chishti (2000) carried out a helminthological survey of freshwater fishes of Kashmir valley and revealed that the fishes of Anchar and Manasbal lakes only were infected with a digenetic trematode *Clionostomum Leidy,* 1856. Cribb, *et al.,* (2000) reported a heterogeneous distribution of *Pomphorhynchus heronsis* in the coral reef fish over small distances. Individual fish from the reef slope had 0-9 worms while as individual from reef flat had 1-122 worms. Other variables (year, season, size) of fish made little contribution to the variation. These results imply both that the fish have very limited local movement and that transmission of the parasite is concentrated locally.

Jahan, *et al.,* (2000) studied the parasites of *Schizothorax* species and *Cyprinus carpio* from River Jhelum and recorded the presence of a new cestode *Bothriocephal*us (Rudolphi; 1808). Machado, *et al.,* (2000) examined the fish for helminth parasites. Prevalence and total host length were positively correlated in fish parasitsed by cestodes. Infection intensity and host length were positively correlated only for the cestode *P. microscopicus*. There were significant differences in the prevalence of parasites in males and females of *C. monoculus.* Cave, *et al.,* (2001) compared the helminth parasite communities in eel from lagoons of Adriatic coast and Tyrrhenian coast. It was proved that there is similarity in composition and structure of helminth communities in eels from coastal lagoons throughout Europe.

Dezfuli, *et al., (2001)* studied species co occurrences and interspecific associations between intensity of infection in helminth communities of three populations of brown trout from northern Italy. Variations in fish size and its effect on infection levels, and whether or not two helminth species used the same or different intermediate host could not be revealed as reported by other authors. Therefore, they

suggested that interspecific associations may be condition dependent: even in apparently similar localities, the same combinations of helminthes species show different associations.

Evans, *et al.,* (2001) gave first record of the *Pomphorhynchus laevis (*Acanthocephala) in fishes from Northern Ireland. Guillen-Hernandez and Whitfield (2001) revealed the sympatric occurrence of the freshwater and marine/estuarine strains of the *Pomphorhynchus laevis* to compare their infection levels in *Platichthys flesus*. They observed that freshwater worms were larger and had more eggs than marine/estuarine worms.

Lyndon and Kennedy (2001) worked on acanthocephalan parasites in freshwater fish from the British Isles. They proposed that all the known species have been able to successfully colonize by a variety of means. Foremost among these is the utilization of a migratory fish host in their life cycle, allowing colonization of new areas and rescue effects in established areas. In addition all six species appear to exhibit resource partitioning by host at either or both the larval stages, thus reducing the potential for competition and further facilitating colonization and survival.

Tingbao and Xianghua (2001) studied the seasonal population dynamics of *Neoecinorhynchus quinghaiensis* in *Gymnocypris species*. Prevalence values were above 44% in all seasons sampled without a distinct seasonal trend-mean intensity reached a peak in the autumn, and then decreased throughout the winter and spring to reach its level in summer. the sex ratio of female to male was both high in winter (1.51:1) and spring(1.48:1).these authors believed that the higher proportion of females and the change in the worm sex ratio in winter can be attributed to the reduced longevity of male worms.

Aloo (2002) conducted a helminthological survey on two fish species and recovered five larval helminth parasites in them: a nematode, an acanthocephalan, *Polyacanthorhynchus kenyensis*, a digenetic trematode and two cestodes. Both prevalence and intensity of the infection of these helminthes increased in large sized fish, whereas male fish were more heavily infected than females. No seasonality in infection was observed by the researcher.

Akifumi, *et al*., (2002) investigated that fish hosts were heavily infected in lakes with dense population of the isopod intermediate host. In rainbow trout, male worms were abundant from winter to spring and female worms were immature during these seasons. Gravid females were abundant during summer and autumn. They

concluded that *Acanthocephalus* sp. is an annual species and its recruitment for the intermediate host to the fish occurs mainly in winter and spring.

Poulin (2002) has examined the relationship between the species diversity of taxa, the mean number of article published per year on each taxon, the mean impact factor of the journals in which they appear. Six taxa of helminths: Nematophora, Acanthocephala, Monogena, trematode, cestoda and Nematoda were considered. Out of these six taxa the mean journal impact factor correlated positively and significantly with the mean annual number of papers published. More number of papers was published on Nematodes, Trematodes, Cestodes than Nematomorphs or Acathocephalans. Amin, *et al*., (2003) gave a description of *Pomphorhynchus spindletruncatus* from freshwater fishes in Northern Iraq and keys to genera of the Pomphorhynchidae and the specie of *P. monticelli,* 1905.

Blanco, *et al.,* (2003) described the best management practices, in aquaculture which control infectious diseases and improves safety of the fish products. These authors stress on the implementation of integrated measures at the production level. Nedeva, *et al., (2003)* studied morphology particularly the morphometry of *Pomphorhynchus laevis* from river Danube. Extensity and intensity of the invasion in different fish hosts species were also investigated. Ziolkowska and Rokicki (2003) searched the real intermediate host of *Pomphorhynchus laevis* in brackish waters of the Baltic Sea. They concluded that *Gammarus zadolachi* is probably an intermediate host for *P. laevis* in the Baltic Sea.

Rauque, *et al.,* (2006) investigated the seasonality of recruitment and reproduction of *Acanthocephalus tumescens* at the component population level. Overall prevalence, mean intensity, and coefficient of dispersion showed the same pattern of seasonal changes. The seasonal feeding pattern of fishes affects the occurrence of *A. tumescens* producing 1 peak in spring and the other peak in autumn. The low temperature in winter delay reproductive process after the autumn periods of recruitment.

Simkova, *et al.,* (2006) investigated the patterns and likely processes connected with evolution of host specificity in congeneric monogeneans parasitizing fish species of the Cyprinidae. They confirmed the hypothesis of specialization i.e. specialist parasites with larger anchors tend to live on fish species with larger body size and greater longevity. The mapping of morphological characters of the attachment organ onto the parasite phylogenetic tree reveals that morphological evolution of the characters of attachment organ is connected with host specificity in

the context of fish relatedness, especially at the level of host sub families. Benesh, *et al.,* (2007) examined the life cycle pattern of acanthocephalans and concluded the various approximate factors that play an important role for its completion.

Mustafa and Altunel (2007) examined three fish species from Enne Dam lake of Turkey for parasitic infections. There was a significant positive correlation between fish length, fish weight, infection rate in Crucian carp but there was no clear correlation existing between length, weight, and parasite infections in bleak. In addition, a significant negative correlation was found between water temperature and infections in golden carp.

Ahmad, *et al.,* (2008) undertook a study to find out the host specificity among the parasites of freshwater fishes of Kashmir and concluded that host specificity of *Rhabdochona guptai* and *Allocreadium nemachilus* was highly specific while as *Pomphorhynchus kashmirensis* showed least host specificity.

Benesh, *et al.,* (2008) recorded five traits from isopods infected with an acanthocephalan (*A. lucii)* and suggested that the host behavior tremendously changes over time with the acanthocephalan infection.

Custodio, *et al.,* (2008) conducted research work for the first time on metazoan parasites of common carp from the river Limpopo and the lagoon Chuali. Nine metazoan parasites were detected including one acanthocephalan (*Acanthogyrus tilapiae*). The parasites communities from river Limpopo and Lagoon Chuali were very similar, exhibiting low diversity and were dominated by a single species, *Pomphorhynchus samfya.*

Ehab and Faisal (2008) studied Largemouth bass *Micropterus salmoides*(L.) which is a popular freshwater spottfish in Michigan. These authors noticed that this fish is severely plagued with endoparasites especially the bass tapeworm, *Proteocephalus ambloplitis* Leidy and different species of acanthocephalans. They observed an inverse correlation: when the number of *Proteocephalus ambloplitis* significantly increased in the ovary and the number of *Neoecinorhynchus* sp. decreased in the intestines. Acanthocephalan adults were found in the intestines or in the pyloric cecae and were usually associated with damage of intestinal mucosa at sites of attachment.

Hermida, *et al.,* (2008) investigated the gills, digestive tract and swimbladder of eels from Ria de Aveiro for the presence of the parasites. Fifteen metazoan parasite species were found including the acanthocephalans parasitizing the fish host.

Rubio, *et al*., (2008) worked on the farmed rainbow trout *Oncorhynchus myskiss* and brown trout *Salmo trutta*. These hosts were monitored for infection with the blood feeding gill fluke *Discocotyle sagittata*. They observed new infections were dominated during summer/autumn and was negligible during winter/spring season. Thus, they again showed temperature is having a vital role in parasitic infection.

Selda, *et al.,* (2008) investigated monthly variations and the effects of host size on parasite prevalence and mean intensity in common carp from Beysehir Lake in Turkey.

2.2) Immunology and biochemistry of Parasites

Bisset (1948) worked on the immune system of fishes and concluded that infection increases with a steep rise in temperature. Summerfelt (1966) described the various forms of immunoglobulins of fish and their structure and functions with respect to higher vertebrates. Alien and Mc Donial (1973) investigated the humoral immune response of fishes and concluded that temperature shows a positive correlation with the parasitic infection and hence the antibody formation. Bradford (1976) has given a sensitive method for quantification of proteins at microgram level by using specific protein dyes. Petit, *et al.,* (1981) studied an ideal diagnostic method of the detection of the parasite surface antigens to accurately determine the level and intensity of the infection. The approach, however, was unsuccessful while determination of the circulating antigens in infected animals.

Parkhouse, *et al.,* (1987) studied the protective effect of nematode antigens and concluded that somatic antigens are poorly immunogenic compared to excretory antigens.

Bunyatova and Elchiev (1989) constructed electrophoretic spectrum of proteins of an acanthocephalan *Leptorhynchoides plagicephalus* and the proteins in the blood serum and liver of *Acipenser stellatus.* The PAGE showed that this parasite induced changes in the composition of blood serum and liver proteins of its host fish. Kennedy and Qureshi (1989) used radioimmunuprecipitation with defined rabbit antiserum and SDS-PAGE and found that there is a significant antigenic similarity between the secreted and somatic antigens of the three nematodes viz. *Ascaris lumbricoides, Toxocara canis and Ascaris. suum.*

Woo and Thomas (1991) compared polypeptide and antigen profile of *Cryptobia salmositica* (virulent and avirulent strains*), C. bullocki* and *C. catostomi* by using SDS-PAGE and Western immunoblot. The avirulent strains of *C. salmositica* had 5 fewer bands than the virulent strain (21 bands) which is, perhaps related to loss

of virulence. *C. catastomi* had the highest number of bands and a different banding pattern than the other two species. The authors tentatively suggested that pathogenic and non pathogenic species of *Cryptobia* have different polypeptide profiles and are antigenically distinct.

Lorenzen (1993) amalgamated the information on the immune response in individual fish with theoretical considerations of its population consequences. Different approaches to the detection of acquired immunity in wild fish were discussed. Roubal (1993) studied the comparative histopathology of *Longicollum* (Acanthocephala: Pomphorhynchidae) infecting the alimentary tract spleen of *Acanthoparus autralis* (Pisces: Sparidae). It was observed that the entire gut wall of the host had been penetrated by the neck and proboscis of the parasite. Also a layer of compact, rounded fibroblasts and scattered connective tissue fibers were surrounding the neck and proboscis of the parasite.

Feng and Woo (1996) produced a mono clonal antibody (IgG1) against the pathogenic haemoflagellate *Cryptobia salmositica*. They observed that this antibody is a protective monoclonal antibody and the antigen it recognizes is located on the surface membrane of *S. salmocitica*. The antibody also inhibits multiplication and affects viability of the parasite under in vitro conditions.

Dezfuli, *et al.,* (1997) studied the ultra structure of the cement apparatus namely cement glands and cement ducts by light and electron microscopy of Pomphorhynchus laevis. Besides, the nature of the secretory product of the cement glands was investigated by histological and electrophoretic methods.

Udo (1998) has explained various types of biochemical techniques, their applications, limitations etc.

Joshi and Singh (1999) characterized two low molecular weight protective antigens of *Haemonchus contortus* by using affinity chromatography and SDS-PAGE technique.

Buchman Kurt (2000) represents a key note paper in which he explains how a range of parasites are exploiting the antiparasitic response mechanisms of the host (teleost) to optimize host- finding, invasion and survival in the host. Some monogeneans cestodes, digeneans, acanthocephalans are even able to resist pronounced cellular host reactions which enables them to firmly attach to the host tissues. However, the author believes that despite these evading mechanisms in the parasites, some parasites are actively rejected by their potential hosts provided these are effectively immunized at certain early points before infection.

Coscia and Oreste (2000) investigated the presence of antibodies against protein antigens of the nematode parasite *Pseudoterranova decipiens* in the plasma and bile of the Antartic Teleost, *Trematomus bernachii*. Three different *P. decipiens* protein solution were prepared: excreted/secreted proteins from live larvae (ESP); surface associated protein (SAP) and cuticular soluble proteins (CSP). Using different immunoassays, these preparations were tested for their ability to bind fish antibody and the specific antibody binding activity was higher in SAP than in CSP. Furthermore, bile antibodies were found to be more reactive and more heterogeneous than plasma.

Knopf, *et al*., (2000) studied the humoral response of the European eel Anguilla *Anguilla* elicited by an experimental infection with a swim bladder nematode *Anguillicola crassus*. Specific antibodies against *A. crassus* in the peripheral blood of the eels were measured using an ELISA and immunoblot technique. The late appearance of antibodies in the peripheral blood supported the hypothesis that the adult parasites elicit the production of specific antibodies and not the invading $L_{3.}$A stage specific antibody response against the $L_3$was not observed. It was thus proved that main antigens are located in the body wall, especially in the gelatinous outer cuticle, of adult *A. Crassus*.

Noga, *et al.,* (2000) purified antimicrobial proteins from rainbow trouts and sunshine bass by RP-HPLC and SDS-PAGE. Mass spectrometry and amino acid sequence data suggested that these proteins are closely related to histone H2B and histone H1. Their findings proved further that histone like proteins may be important defensive molecules in fish.

Saifullah, *et al., (*2000) worked on the excretory /secretory (ES) metabolic products released by *Gastrothylax crumonifer* (trematode:Digenea) during in vitro incubations and the somatic extract of the adult parasites were analyzed using PAGE. Immunogenicity of ES and somatic extracts were evaluated by immunoblotting and ELISA using sera against ES and somatic antigens in rabbits. It was confirmed that when the ES antigens were allowed to react with antisomatic extracts in hyper immune sera, the titer of IgG increased up to a dilution of 1: 128000.

Buchmann, *et al.,* (2001) studied the humoral immune response in fishes sought out the best possible ways for vaccine making against these fish parasites.

Dudinak and Snabel (2001) studied the morphological and genetic variability of three *Pomphorhynchus laevis* populations obtained from the Slovak and Czech Republics and two fish hosts were examined for the purpose. They observed a close

relationship between the parasite body length and size of the fish host. Besides, morphological characters of prime importance included a number of hooks in a row and number of rows of hooks in proboscis. The results from isoenzyme analysis were congruent with morphological data in showing clear differences in the genetic constitution of Slovak and Czech populations.

Herrero and Gomez (2001) carried out hypersensitivity prick tests against shellfish and airborne allergens. The total serum IgE levels were quantitated, and SDS-PAGE and IgE immunodetection was carried out with both raw and cooked shrimp, Norway lobster, Sea crab (2 species) and prawn antigens.

Jitra (2001) revealed a specific and sensitive immunodiagnostic method for *Opisthorchis viverrini* infection and the corresponding cross reactivity with other helminth infections. Sepharyl S-200 HR gel filtration chromatography was used to have partially purified *Bithynia funiculate* extract as an alternate antigen. Four major peaks for snail extract were obtained vizP_1, P2, P_3 and, P4 respectively. The ELISA was used to evaluate the serodiagnostic potential of these peaks.

Knox and Smith, (2001) isolated gut antigens of GIT nematodes (contortin, H11, H-gal, GP, GPI and cystein proteinases) by chromatographic techniques and used them in vaccination trials against gastro-intestinal nematodes.

Yongsawatdigui, *et al., (2001)* carried out a proteolytic degradation of tropical *Tilapia surimi* by biochemical processes and rheologically characterized it to identify a group of proteinase(s) responsible for its textual degradation. Storage modules (G) of surimi gels mixed with either soybean trypsin inhibitor (SB) or leupeptic (LE) was higher than other inhibitor indicating that serine type proteinase(s) were involved in proteolysis of tropical tilapia.

Hamwood, *et al*., (2002) partially characterized secreted anterior adhesives of some monogeneans parasites of fish. These researchers first performed scanning and transmission electron microscopy and used analytical methods viz SDS-PAGE, High pressure liquid chromatography (HPLC) and Liquid chromatography mass spectroscopy (LCMS).

Wanderly and Narsiza (2002) carried out the cell fractionation procedures of protozoans to study the cellular components in details. Trypanosomatids were first examined by transmission electron microspy and then various components like plasma membrane, flagellum, mitochondrion- kinetoplast complex, golgi complex, glycosome endocytic compartments were isolated. Similarly fractionation of

Apicomplexa was also performed. Its membrane complex were isolated and characterized by SDS-PAGE and western blot techniques.

Feroz, *et al.,* (2003) worked on the humoral immune response of *Schizothorax niger* and *Cyprinus carpio* against helminth infection. They used agar gel diffusion technique to precipitate antibodies against the antigens of *Adenoscolex orein*i and *Pomphorhynchus kashmirensis*. Immunochemical investigations of the antibodies indicated that they resembled Ig M of mammals. They also observed that with a fall in temperature during winter months, the antibody level against helminth parasites went down, suggesting the seasonal variance in helminth infection.

Gupta, *et al*., (2003) purified the *Fasciola gigantica* antigens using affinity chromatography and SDS-PAGE techniques. They observed six antigenic proteins of 27.7to37.5 KDa molecular weights. They coated micrcotitration plate with the sample concentration of 15 µg/ml, detected antibodies in the sera (1:2000 dilutions) of the infected animals as early as 2 weeks post infection (PI) and a peak absorbance value of 0.588 at 14 weeks PI. They concluded that 27.7 to 37.5 KDa antigens have promising diagnostic value.

Rodriguez and Crespo (2003) carried out their work on proteins from soluble extracts of sporocysts of the two the protozoan parasites of cephalopods *Aggregata octopiana* and *A. eberthi.* SDS-PAGE and immunoblotting techniques were used which showed a characteristic electrophoretic pattern and species specific antigens and thus helped in differentiation of species.

Tort, *et al., (*2003) studied the ability of fish to mount successfully immune responses with apparently more robust innate responses than that observed in higher vertebrates.

Wegner, *et al.,* (2003) tested the idea whether the simultaneous infections from multiple parasite species could cause diversification (balancing selection) in resistance genes both at the population and individual level. They tested these ideas in highly polymorphic major histocompatibility complex (MHC) genes from three-spined stickle-baeks. Partial correlation analysis revealed an influence of parasite diversity on MHC class IIB variation whereas general genetic diversity assessed at seven microsatellite loci was not correlated with parasite diversity.

Ahmad, *et al.,* (2004) fractioned the soluble extracts of *Gigantocotyle explanatum*, isolated from the liver of *Bubalus bubalis* on Sephadex G-200 columns. Nine major fractions referred to as F1, F2, F3, F4, F5, F6, F7, F8 and F9 were separated. Each fraction was tested by ELISA for antigenicity using sera from *G.*

explanatum infected field buffaloes. Fractions F1 and F2 were highly antigenic, F3, F4, F6 and F7 were moderately antigenic and F5, F8 and F9 were poorly antigenic. Analyses by SDS–PAGE revealed that each fraction comprised several polypeptide(s) in the molecular weight range of 29 to 205 kDa. Results of Western blotting indicated that not all polypeptides which appeared in the SDS–PAGE were antigenic. The antigenic molecules of each fraction were mostly in the low molecular weight range of 14 to 94 kDa with the polypeptides in the range of 14, 14, 18, 21–25 and 34–36 kDa.

Chibani, *et al.,* (2004) describes the biochemical procedures for identifying the Pomphorhynchus species. Karen, *et al.,* (2004) provided a holistic outlook on adverse reactions to diseased fishes.

Yildiz, *et al.,* (2004) investigated the pathological changes of intestine of fish caused by the infection of the *Pomphorhynchus laevis.* It was shown that it is not only found in the host's alimentary canal but also in extra intestinal spaces. Histological examination revealed that mucosa; sub mucosa and muscle layers were completely damaged at the site of attachment.

Dasgupta, *et al*., (2005) characterized the *Fasciola gigantica* soluble antigens. In an experiment *Fasciola gigantica soluble* antigens were isolated and purified by column chromatography (gel filtration).Two prominent peaks (GP_1 & GP_2) were obtained by ion exchange chromatography, these fractions were further resolved into P_1D_1, P_2D_2, P_1D_3, & P_2D_1, P_2D_2, P_2D_3 respectively.

Megeed (2005) undertook a study on the characterization of *Fasciola gigantic*a partially purified worm antigens and their potency in the diagnosis of fasciolases. Chromatographic analysis of *F. gigantica* adult crude extract was undertaken using Sphadex G-200 and four fractions were isolated by this approach. The isolated fractions showed simple electrophoretic profile as judge by SDS-PAGE, and compared with the complex profile of crude extract.

Revilla, *et al.,* (2005) focused their study on characterization and isolation of *Dicrocoelium dendriticum* antigens or their fractions that could be used for the immunological diagnosis of Dicrocoeliasis. Somatic and excretory-secretory antigens were analyzed by SDS-PAGE and their specificity was evaluated by western blot with homologous and heterologous sera. The antigens were partially purified by chromatographic technique of filteration(Sepharyl S-300) and ion exchange (DEAE Sepharose).western blot analysis using sera of ovine infected with *D. dendriticum* revealed eight main antigenic polypeptides ranging from 24-205 KDa for somatic

antigens and seven for E/S antigens with apparent molecular mass in the range of 26-205 KDa.

Yambot and Yen-ling (2006) investigated the immunization of *Epinephelus coioides* against protozoan parasite *Cryptocaryon irritants*. The host immunoglobulins were characterized by SDS-PAGE. Mucus titer was detected by ELISA.

Cramptom and Vanniasinkam (2007) described the new ways and means for preparing vaccines against the infectious organisms.

Despotovic and Perendija (2007) investigated that certain parasites, especially the intestinal acanthocephalans of fish, can accumulate heavy metals in concentrations much higher than those in tissues of the host or in the aquatic environment. It has supported earlier investigations that acanthocephalans are very useful organisms in biomonitoring of metals in aquatic ecosystems.

Haus and Sures (2007) examined heavy metal concentrations in different hosts-parasite system. They observed that for almost every analyzed metal *Pomphorhynchus laevis* in chub and barbel showed higher heavy metal concentrations than the host tissues, whereas in eel heavy metal concentrations of *P. laevis* are below the concentrations of hosts tissues. They also observed inverse relationship of metal accumulation among the parasites if they showed co occurrence in the same host. Thus the accumulation patterns may vary depending on both the acanthocephalans and the hosts' species. Thus concluded host-parasite system should be regarded as sentinels rather than single organism.

Munir, *et al.,* (2007) characterized outer membrane proteins of *Pasteurella multocida* by using SDS-PAGE technique. Molecular weights of these proteins were determined by plotting graph between R_f value and log of molecular weight. A total of six polypeptides ranging from 15KDa to 91KDa were observed which included two intense bands of 39 and 32KDa, and 4 less intense bands of 91, 72, 44 and 15 KDa.

Nacher and Sures (2007) worked on Barbel, the most abundant fish of river Danube to find whether its parasites could be used as pollution indicators. It was again confirmed that acanthocephalan, especially *Pomphorhynchus laevis* has highest accumulation capacity for heavy metals than the host's tissues (muscle, liver and intestine) or the aquatic environment.

Norouzi, *et al*., (2007) investigated humoral immunity and antigenic pattern of midgut of fed adult female *Hyalomma anatolicum anatolicum*. Antegenic characterization was done by using SDS-PAGE and western blot.

Rubio (2007) focuses on immune responses (humoral and cellular; innate and acquired) of teleost fishes against *Polyopisthocotylean*s and contrasted it to defense mechanisms against *Monopisthocotyleans.*

Sures (2007) had worked on three aspects of fish parasites: parasites as sinks for pollutants within their hosts; parasites as a diagnostic tool to test bioavailability of substances; changes of biomarker responses of the host against pollutants. This research work lucidly describes the interrelation between parasitism and pollution.

Ahanger, *et al.,* (2008) studied the histopathological aspect of *Pomphorhynchus* species infecting the Schizothorax species. The researchers concluded that *Pomphorhynchus s*pecies of Acanthocephala was one of the chiefly responsible fish parasites responsible for mortality and morbidity of its fish host.

Akimasa, *et al.,* (2008) carried out agglutination of somatic antigens of the fish pathogenic ciliate *Cryptocaryon irritants.*

Garcia-Coirades *et al*., (2008) isolated and immuno-localized the putative protective antigen, p26/23 from adult *Haemonchus contortus*. A soluble extract from adult helminths obtained from the abomasa of hyper-infected (12,000 infective larvae) female Manchego lambs and treated with a mixture of protease inhibitors was subjected to affinity chromatography (hexyl glutathione) to eliminate the enzyme-glutathione S-transferase. The eluate was analyzed by electrophoresis under denaturing and reducing conditions (SDS-PAGE), electro-transferred to nylon membranes and assayed by western blot with sera from immunized lambs. The bands recognized by lambs' sera corresponding to proteins with a molecular weight of 23-26kDa (p26/23) were excised, eluted and separated by reverse phase chromatography. This allowed the isolation of single protein which was expressed in both infective larvae (L3) and adult stage of parasite. Immuno-localization studies showed that the protein was expressed in the four hypodermic chords (dorsal, ventral and two lateral) of the nematode. The immune-localization profile of p26/23 in the worm did not correspond with any other *H. contortus* antigens reported to date. The low molecular weight fraction p26/23 obtained from soluble extracts of adult helminths, confer notable protective levels against haemonchosis in sheep.

Juan, *et al.,* (2008) investigated the humoral immune response of the tilapia *Oreoshromis niloticus* against *Cichlidogyrus* spp using a direct ELISA and double immunodiffusion tests. Results showed that tilapia is capable of producing a humoral response against an antigenic extract of *Cichlidogyrus* spp.

Meshgi, *et al.,* (2008) compared electrophoretic patterns of somatic and excretory-secretory antigens of *Fasciola hepatica* and *F.gigantica* by SDS-PAGE. The E/S and somatic antigens were prepared by incubation and homogenizing of adult flukes respectively. The antigens were electrophoresed using SDS-PAGE. *F. gigantica* had 11 major protein bands with molecular weights of 18, 22, 24, 33, 36, 42, 46, 57, 60, 62, 68 KDa, whereas F. hepatica had proteins characterized by 8 distinct bands with molecular weights of 18, 22, 24,33,36,42,33,36,42,46 and 62KDa.

Rolbiecki and Rolbiecki (2008) gave a detailed account of metazoan parasites of the Lumpsucker inhabiting shallow coastal areas of Northern Atlantic.

Sures (2008) gave a mini review on the effects of pollution on the occurrence and distribution of parasites. This paper presents some promising examples of interdisciplinary studies paying attention to the fact that under natural conditions no organisms will only be affected by either parasites or pollution.

Takashi, *et al.,* (2008) focused on the molecular innate immune mechanism in the teleost fish in reference to the known mammalian system, the research work also includes future perspectives on how this basic knowledge, coupled with existing molecular biological techniques, can contribute to the control of fish diseases.

Witek and Herlyn (2008) worked on Syndermata phylogeny for reconstructing the evolution of the acanthocephalan endoparasitism. Mapping morphological character evolution onto molecular phylogeny suggests the reduction of the corona and the emergence of a retractable anterior end (rostrum, proboscis) before the separation of Acanthocephala. In other words, the evolution of rostrum might have been a key event leading to the latter evolution of the acanthocephalan endoparasitism.

Ventura, *et al.,* (2008) estimated the complexities caused by *Anisakis simplex* parasite present in fish. They performed patch tests with live, cooked and frozen larvae and all of them induced sensitization. They also emphasized that *A. simplex i*s responsible for both intermediate allergic reactions and cell mediated reactions. They explained these differences due to allergenic proteins present

Ananda, *et al.,* (2009) prepared somatic and metabolic antigens from *Echinococcus granulosa* adult worm to detect their specific antibodies in dogs by indirect ELISA.

Buron, *et al*., (2009) studied the bioaccumulation of metals by helminths. They optimized an in vitro culture technique of the acanthocephalan *M. monoliformis*

and initiated in vitro exposure to metals. Helminth parasites showed variations in their abilities to sequester various metals. Adult acanthocephalans were particularly efficient as bioacccumulators of heavy metals.

Singh and Mishra (2009) reported the results from computational characterization of merozite surface protein (MSP1), precursor screening of highest scoring potential CTL epitopes for 1712 overlapping peptides binding to thirty four HLA class-1 alleles and twelve HLA class-1 supertypes using bioinformatics tools.

Ritu, *et al.,* (2010) studied polypeptide profile of somatic antigen of *Paramphistomum epiclitum* (PSAg) and *Gastrothylax crume-nifer* (GSAg) by SDS-PAGE. PSAg and GSAg showed 14 and 19 polypeptides in the range of 14.9-95.5 and 13.7-129.6 kDa with six common polypeptides of mol wt 16.8, 21.8, 23.7, 35.5, 43.4 and 70.8 kDa. *P. epiclitum* experimentally infected sheep sera were used for identification of specific immuno-dominant peptide in the range of 37^10 kDa against *P. epiclitum* by western blotting. Hyperimmune sera (HIS) was raised in rabbit against the identified polypeptide, IgG was separated from HIS and an immunoaffinity column was constructed with a binding percentage of 83.74 of IgG with CNBr activated Sepharose 4B. Purification of somatic antigen (PSAg) was done with immunoaffinity chromatography and 37^10 kDa protein antigens were isolated in pure form with recovery percentage of 2.97%.

Soad, *et al.,* (2010) worked on isolation of immunodiagnostic fraction from *Strongylus vulgaris* adult worms by CNBr-Sepharose 4B affinity column chromatography. The isolation process resulted in a fraction with 2161.5 fold increase in binding activity compared to its crude extract. Characterization of the isolated fraction by SDS polyacrylamide gel electrophoresis and isoelectric focusing showed that the fraction consists of only two bands of 39 and 31 KDa with isoelectric points of 6.8 and 6.7. Comparative evaluation of the immunogenic binding activities of the crude extract, unbound and bound fractions by ELISA proved the potency of the bound fraction over the other two antigens. Diagnosis of *S. vulgaris* infection in horses by ELISA in which the bound fraction was utilized, recorded high infection percentage (73.7%) as compared with the parasitological examination (27.6%).

A perusal of the above reviewed literature clearly indicates that no work so far has been undertaken on the isolation and characterization of parasites in the Kashmir Valley and on the epidemiological studies of *Pomphorhynchus kashmirensis*. The present endeavor opens the new era of research for the control of helminth parasites and makes a foundation for new researchers to devise vaccines against these dreadful parasites. With this background the present study was undertaken.

Collection site: River Jhelum near Rajbagh

Showing Collection site River Jhelum near Rajbagh

Collection site: Dal Lake near Nigeen

Collection site Dal Lake near Hazratbal

CHAPTER: 3

MATERIALS & METHODS

The present work mainly deals with studying the prevalence, protein profile and the antigenicity of *Pomphorhynchus kashmirensis* and as such is of diverse nature. Therefore, separate methodological considerations were adopted to fulfil the aims and objectives of study. These include:

3.1. To study the prevalence of *Pomphorhynchus kashmirensis* in fishes of Dal Lake and River Jhelum.

3.2. To study the antigenic profile of *Pomphorhynchus* somatic extract.

3.3. To detect the antigenicity of purified antigens.

3.1. Prevalence of *Pomphorhynchus kashmirensis* in fishes of Dal Lake and River Jhelum:

3.1.1. Collection of the hosts

Schizothorax fishes were collected from Dal Lake and River Jhelum at different study sites with the help of a local fisherman. Study sites of Dal Lake include Nigeen, and Hazratbal. From River Jhelum fishes were collected from Chattabal and Rajbagh. The fishes were brought alive or fresh to the Parasitological Research laboratory I, PG Department of Zoology, University of Kashmir, Srinagar.

3.1.2. Parasite collection

The fishes were examined for the endoparasites by killing them by the usual method of a blow on the head. Fishes were dissected & body cavity was thoroughly examined

for any parasite. Intestines were placed in Petri dish containing normal saline (0.75%Nacl, Cable 1958) to allow adhering parasites to be released from the lumen. In case of acanthocephalans, if the anterior end was deeply bored in the mucosa of the intestine, a few crystals of the methanol were added to the normal saline, containing the parasites adhered to the intestinal wall. This led to immobilization of the parasites & loosening of the grip on the intestinal wall & facilitated the detachment of proboscis in case of acanthocephalans without causing any distortion in the arrangement of hooks. The regular record of the collection was maintained and the prevalence of *Pomphorhynchus kashmirensis* was carried out by the following formula:

3.1.2. a.

$$\text{Prevalence} = \frac{\textit{total number of hosts infected}}{\textit{total number of hosts examined}} \times 100$$

Prevalence is the percentile representation of infected hosts divided by hosts examined multiplied by 100.

3.1.2.b. Statistical analysis

The whole data was fed into a Microsoft Excel 2010. A computer program (SPSS 10.05 for windows) was used for data analysis. Student's t-test was used for the analytical assessment. The differences were considered to be significant when the p-value obtained was less than 0.05.

3.2 To study the antigenic profile of *Pomphorhynchus* somatic extract:

3.2.1 Preparation of the PBS antigens

3.2.1. a. Preparation of Phospho buffer saline (PBS)

Reagents required:137mM NaCl, 27mM KCl, 10mM Na_2HPO_4, 1.8 mM KH_2PO_4.Quantity of all these reagents is taken as per the following formula for 1 litre (1000ml) of PBS.

Mass of the reagent /1000 X Molecular weight/1000 X 1000

i) 137 mM NaCl : 8.0008 g.

ii) 27 mM KCl : 2.01 g.

iii) 10 mM Na_2HPO_4 : 1.41 g.

iv) 1.8mM KH_2PO_4 : 0.28 g.

Dissolve the calculated quantities of these reagents in 1000 ml of double distilled water. Then adjust the pH 7.2.

Whole worm antigens of *Pomphorhynchus kashmirensis* were prepared by dissolving 2 grams of parasites in 100ml of the PBS (pH 7.2) and homogenized at 12000 rpms for 15 minutes. The homogenate was kept in refrigerator at overnight & then centrifuged at 6000 rpm for 30 minutes. The clear supernatant was collected in small tubes as a purified PBS antigen & stored at -20 ^{o}C.

3.2.2. Estimation of protein concentration by Lowry Method

This assay was introduced by O.H Lowry and his co-workers in 1951.It is a highly sensitive method and can detect proteins as low as 5µl/ml. This is the most widely used method for protein estimation. (Zargar, *et al.*, 2000)

3.2.2. a.Reagents required

1) Cu-reagent

a) 4% sodium carbonate : 4g $100ml^{-1}$

b) 4% Sodium Potassium Tartorate : 4g $100ml^{-1}$

c) 2% Copper sulphate : 2g $100ml^{-1}$

These components are mixed in the ratio of 100:1:1 at the time of experiment. In order to avoid precipitation, the solution "b" is added to solution "a" followed by "c".

2) Follin's reagent: **(Follin and Ciocaltea, 1972)**

Prepared solution of Banglore Genae was used. The Stock solution was diluted in the ratio of 1:4 by distilled water.

3) Standard BSA Solution: 50mg of BSA was dissolved in 100ml distilled water.

Table.1: TESTING PROTOCOL

S. No.	Solution (ml)	Test tube no. (BSA)									Sample
		B	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**U1**	**U2**
1	Protein solution	-	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.2	0.4
2	Dist. water	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.8	0.6
Mixed											
3	Copper reagent	5	5	5	5	5	5	5	5	5	5
Mixed well and incubated for 10 minutes											
4	Folin's reagent	1	1	1	1	1	1	1	1	1	1
Incubated for 30 minutes at room temperature											
5	At 700nm	-	0.15	0.27	0.35	0.40	0.54	0.65	0.78	A1	A2
6	Amount of protein (mg)	-	0.05	0.10	0.15	0.20	0.25	0.30	0.35	XI	X2

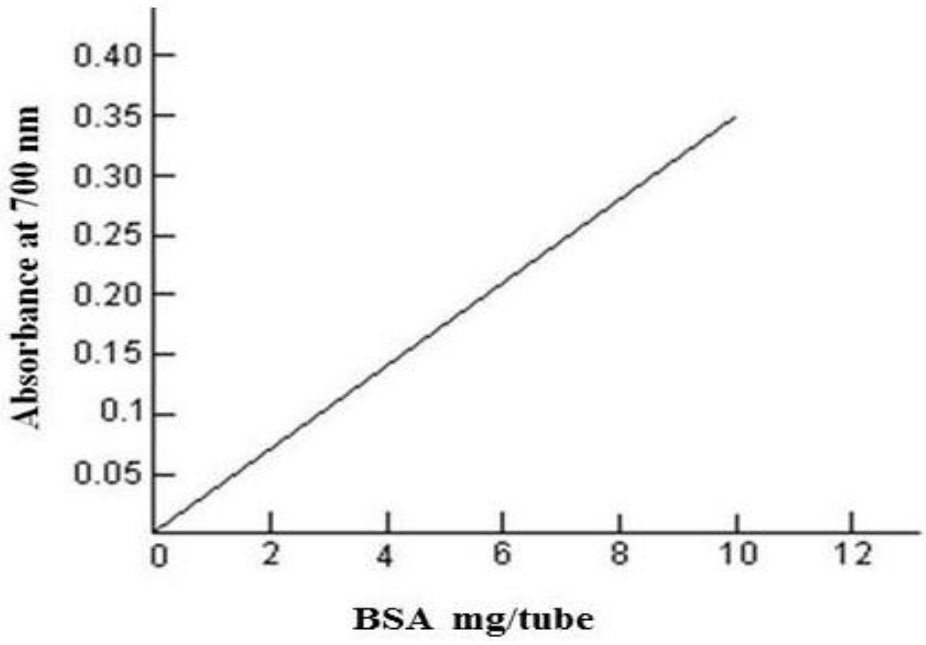


Figure 3. A typical standard curve obtained using BSA as standard

3.2.3. Production of antisera

Healthy rabbits were used for raising hyper immune sera against crude somatic antigens of *Pomphorhynchus kashmirensis.* Crude PBS antigens containing one mg protein in equal amount of Freund's complete adjuvant was thoroughly mixed with the help of syringes till white precipitate was observed. The following immunizations were given intramuscularly on day 15^{th}, 30^{th}, 45^{th} and 60^{th} after the first inoculation along with Freund's complete adjuvant. After 15^{th} day of last immunization, the animals were bled and sera samples were collected.

3.2.4. Isolation of somatic antigens from *Pomphorhynchus kashmirensis* through Affinity chromatography.

3.2.4.a. Construction of immuno affinity column:

Sera from infected fish were utilized as a source of antibodies for preparation of affinity column. Blood from 6 randomly selected infected fish was collected in non-heparinised tubes and allowed to coagulate for 30min at room temperature, following which the tubes were centrifuged at 10,000rpm at 4 ^{o}C for 10min. Sera was separated from the cell pellet and stored at -20^{o}C until further use.

For construction of affinity column, Immunoglobulins (IgG) were precipitated from the Fish sera with ammonium sulphate as per the method of Fey et al. (1976). The precipitated Igs were extensively dialyzed against coupling buffer i.e. 0.1 M NaHCO3, 0.5 M NaCl, pH 8.5 for 36 h and coupled to swelled CNBr activated Sepharose-4.

3.2.4.b. Affinity purification of antigen

A total of 28 mg equilibrated (20 mM Tris saline, pH 8.0) PSAg (*P. kashmirensis* somatic antigen) was loaded on the pre-equilibrated affinity column and then washed with excess equilibrating buffer. The bound proteins were eluted using 0.2 M Glycine HCl (pH 2.2) and the pH of the eluted fractions was brought to neutral by adding 2 M Tris. The absorbance of fractions was measured at 260 and 280 nm on a spectrophotometer (Photometer 5010) and the protein concentration was estimated (Aiken and Learmoth 1996). The column was regenerated using 0.1 M Tris–HCl, 0.5 M NaCl, pH 8.5, 0.1 M Sodium acetate and 0.5 M NaCl, pH 4.5 after each use. The eluted bound protein was dialyzed extensively against 20 mM Tris-saline buffer, pH 7.4, concentrated with PEG 20,000 and was designated as affinity purified *P.*

kashmirensis somatic antigen (Aff-PSAg). The protein content of the antigen was determined spectrophotometrically (Aiken and Learmoth 1996).

3.2.5. Characterisation of affinity purified *P. kashmirensis* somatic antigen (Aff-PSAg) antigens by SDS PAGE.

SDS polyacrylamide gel electrophoresis involves the separation of proteins based on their size. By heating the sample under denaturing and reducing conditions, proteins become unfolded and coated with SDS detergent molecules, acquiring a high net negative charge that is proportion to the length of the polypeptide chain. When loaded onto a gel matrix and placed in an electric field, the negatively charged protein molecules migrate towards the positively charged electrode and are separated by a molecular sieving effect. After visualization by a protein specific staining technique, the size of a protein can be estimated by comparison of its migration distance with that of a standard of known molecular weight.

3.2.5. a. Reagents required:

a)Acrylamide solution (30%):

Acrylamide : 29 g

Bis acrylamide : 1g

Dissolve the solution in 70 ml of distilled water to make 100ml.

b) 1.5M Tris (ph8.8):18.17gms

The 18.7 gms of Tris were dissolved in distilled water & the final volume was made 100ml.

c)1M Tris (PH 6.8) solution:

Tris : 12.11 gms

Distilled water : 80 ml

d) 10% SDS: 10 grams in 100 ml of Distilled water.

e) 10% Ammonium persulphate (polymerising agent): 10 grams of APS were dissolved in 100ml of Distilled water and covered with Almunium Foil and stored in darkness at -20 ^{o}C.

f) TEMED prepared solution: TEMED was added at the time of loading the gel.

1.5 M Tris buffer is used in Resolving gel. 1 M Tris buffer is used in stacking gel. For the present study 8% Resolving and 5%Stacking gel is used. These were prepared by mixing the above solutions as per the following protocol.

Table.2: Solutions for preparing 8% Resolving gel of 5ml

SOLUTION COMPONENTS	5ml
Water (ml)	2.6
30%Acrylamide mixture	1.0
Tris1.5M (pH 8.8)	1.3
10%SDS	0.05
10%Ammonium per sulphate	0.05
TEMED (ml)	0.004

Table.3: Solutions for preparing 5% stacking gel

Solution Components	Volumes (ml) per gel mold volume of 5 ml
Water (ml)	3.4
30% Acrylamide mixture	0.83
1M Tris(ph6.8)	0.63
10% SDS	0.05
10% APS	0.05
TEMED (ml)	0.005

g) Loading buffer:

2mM Tris Hcl	:	1.514 gms
Glycine	:	7.205 gms
SDS	:	0.50 gms

Mix 2mM Tris in 490 ml of Distilled water. Add glycine to it and shake till glycine mixes fully in Tris and then add SDS and mix.

h) Sample Buffer:

1M Tris (PH 6.8) solution	:	12.11 gms
Distilled water	:	80 ml
SDS	:	1.5 gms
Bromophenol blue	:	0.002gms

Glycerol was added to make the final volume 12.5ml then 150 µl of β- beta mercaptoethanol was added to 1 ml aliquote. Sample buffer is stored at -20 ^{o}C in 1ml aliquot. At the time of loading of sample 4µl of sample were mixed with 1 µl of sample buffer and the sample solution were loaded in the wells of gel. This running buffer used was 5x.

The slabs, spacers, comb and assembly were washed thoroughly with distilled water. After setting the apparatus, the sealing was done with 1% agar. 8% separating gels were prepared as above & poured in between the plates immediately. The top of the separating gel was overlaid with 1ml of distilled water in order to reduce the surface tension. The gel was left for one hour for polymerization. After polymerization, water was removed & the stacking gel prepared was poured over the separating gel. The plastic comb was inserted immediately to form the wells & the glass slab was kept undisturbed for polymerization

3.2.5. b. Loading of samples

Loading buffer and protein solution were mixed in the ratio of 1:1in a tube. The tube was then placed in boiling water for about 3 minutes.

3.2.5. c. Electrophoretic run:

30(ml) of protein sample was loaded in each well.15 µl molecular weight marker (sigma) was also loaded in separate lane. After loading, the gel was run at room temperature. A constant current of 14mA/h (320 V) was maintained during the migration of proteins through staking & separating gels. When the tracking dye (bromophenol blue) reached approximately up to the end of gel the run was stopped.

3.2.5. d. Staining and destaining of gels

After the electrophoresis, the gel was removed carefully and placed in a petridish containing staining solution prepared by dissolving 25g of Coomassie blue(R-250) in a mixture of 54ml of 50% methanol & 46ml of acetic acid. The staining was done at room temperature for two hours. The gels were then removed from staining solution, rinsed with distilled water & placed in the destaining solution containing 10 ml of acetic acid, 10ml of methanol and 80ml of water. Then the gel was observed for protein bands and compared with known molecular marker (Genei Cat #PMWB 105975).

3.3. To detect the antigenicity of purified antigens

3.3.1. Immunodiagnostic methods

To measure the antigenicity of the purified antigens the commonly used serotest viz., Ouchterlony double diffusion (ODD) was used.

Ouchterlony double diffusion (ODD)

Crude somatic and partially purified pooled fractions of *Pomphorhynchus kashmirensis* were subjected to double immunodiffusion against rabbit hyper immune sera. The ODD was performed according to the method of Hudson and Hay (1989). 2% agar powder was dissolved in 100 ml of Barbitone buffer Ph 8.2, the composition of which is

a) Barbituric acid : 1.83g
b) Sodium barbitone : 10.6g
c) Distilled water : 1 litre
d) Theomersol : 2-3 drops

With the help of pipette, grease free glass slides precoated with 5 ml of hot gel were kept undisturbed at room temperature. After solidification a central well was punctured using gel punching template. Keeping a distance of 5mm, wells of 4mm diameter were made around the central well and then sealed at bottom with 0.3% agarose to prevent leakage. The central well was loaded with 15µl antigen sample and the peripheral wells with test sera (15µl).The slides were then incubated at 37^0C in a moist chamber for 24-48 hours for the development of precipitation lines. Staining of the lines was carried out by Coomassie brilliant blue(R-250) and Amido Black. After staining the gel slides were washed and then observed.

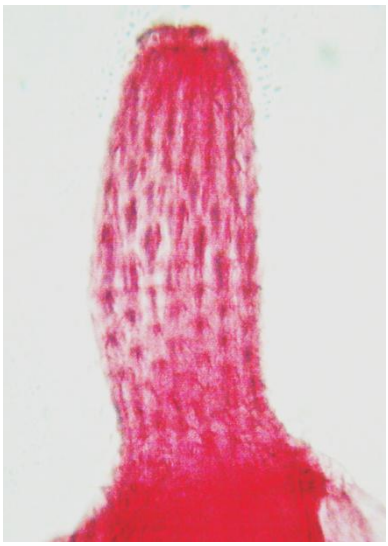
Showing fishes caught by local fisherman at Chattabal

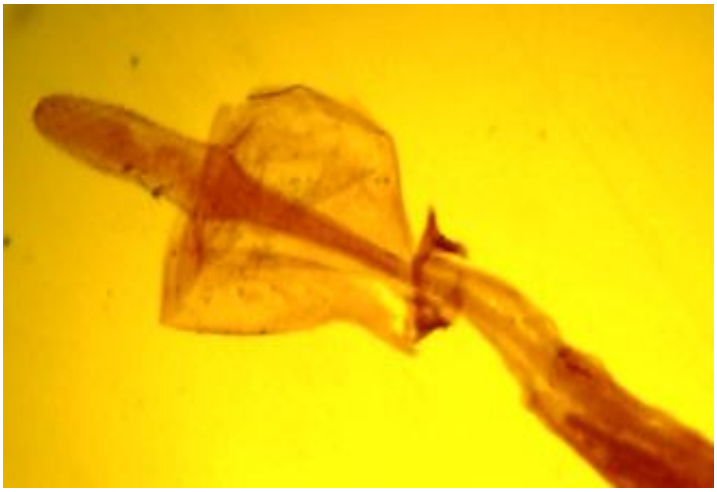
Showing various species of Schizothorax.

Showing *Schizothorax curvifrons.*

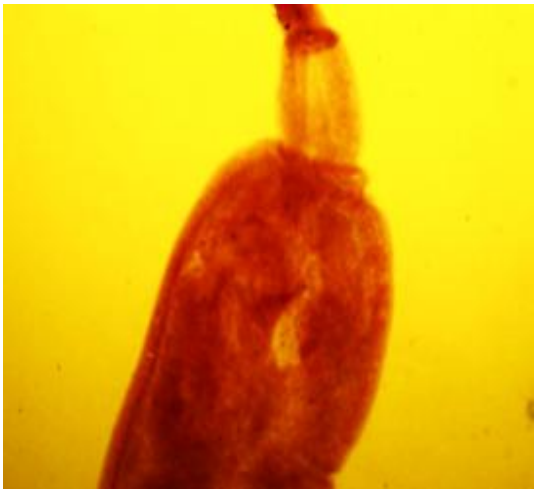
Showing *Schizothorax niger*

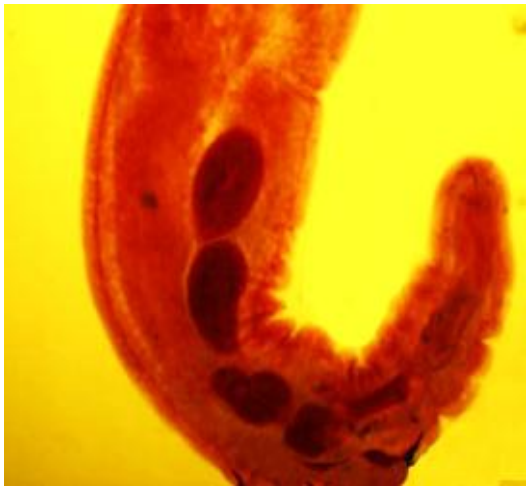
Showing *Schizothorax esocinus*



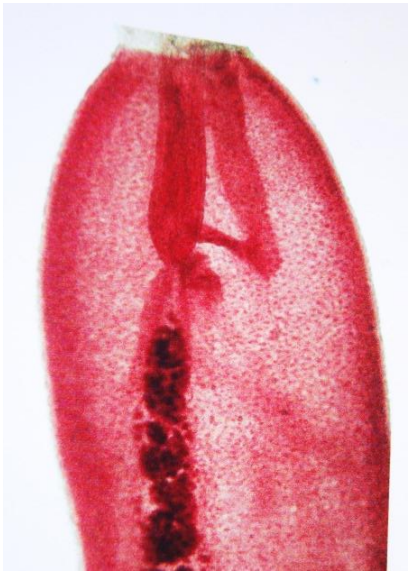


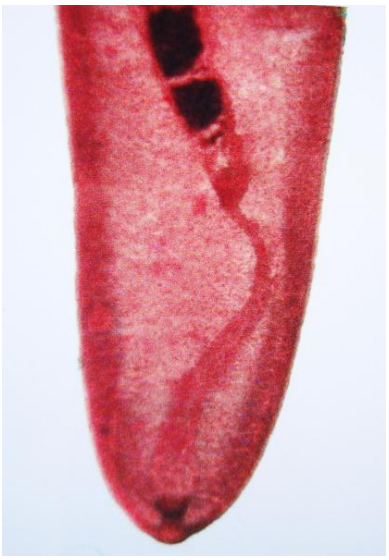
Anterior end *of Pomphorhynchus kashmirensis*





Middle and Posterior end of Male *Pomphorhynchus kashmirensis*





Middle and Posterior end of Female *Pomphorhynchus kashmirensis*

CHAPTER: 4

RESULTS & DISCUSSION

Present study was aimed at studying the isolation and characterization of *Pomphorhynchus kashmirensis* somatic antigens. For this purpose, various species of *Schizothorax* viz., *S. niger, S. esocinus, and S. curvifrons* were collected from the Dal Lake and River Jhelum and then scanned for the parasites. The nature of somatic antigens was observed through various laboratory techniques from December 2009 to November 2010. For a clear understanding, the observations have been divided into following sub headings dealing with various aspects of the study.

4.1. Prevalence of the *Pomphorhynchus kashmirensis* in Dal Lake and River Jhelum

4.2.Purification of the somatic antigens of *Pomphorhynchus kashmirensis*

4.1.Prevalence of *Pomphorhynchus kashmirensis* in Dal Lake and River Jhelum:

Prevalence studies of *Pomphorhynchus kashmirensis* is the first step of the present research work. In fact it provides the basic foundation for any parasite control measures. A total of 363 fish specimens of *Schizothorax* species were collected and out of which 203 fishes were collected and examined from Dal Lake and 160 fishes were collected and examined from River Jhelum during the present study. On examining 363 fish specimens 94 were found to harbor the *Pomphorhynchus kashmirensis* parasite constituting an overall prevalence of 25.89%. Out of 203 specimens examined from the Dal Lake only 42 specimens were found infected with

the *Pomphorhynchus kashmirensis* which constitutes the prevalence of 20.68%. Similarly out of 160 specimens examined from the River Jhelum only 52 specimens were infected with the *Pomphorhynchus kashmirensis* which constitutes 32.5% prevalence (Table 4). Infection patterns of Pomphorhynchus were greatly influenced by seasonal variance, fish species and type of water body. It was seen that overall prevalence *Pomphorhynchus* was low which is in accordance to the studies done by Spall and Summerfelt (1969) and Chishti and Peerzada (1998) who showed 0.7% and9.3% infection of acanthocephalan parasites respectively. The low prevalence might be due to low availability or consumption of intermediate hosts. Seasonal variation in incidence of helminth parasitism in fishes was probably influenced by the annual life cycle of the parasites.

Also, *Pomphorhynchus kashmirensis* showed a wide host range and was successfully establishing in various species of Schizothorax. The highest prevalence was found in *S. niger* (30 %) (26.19% in Dal and 34.85% in Jhelum) followed by *S. curvifrons* (27.11 %) (19.11% in Dal and 38% in Jhelum) and least prevalence was found in *S. esocinus* (17.89%) (13.72% in Dal and 22.73% in Jhelum). The findings of the present results are in accordance with the studies of Ahmad, *et al.*,(2008) (Table:4, Fig.4 and fig.5). However the overall prevalence of 25.89% can be attributed to various factors like temperature and availability of food. The host species generally shows a minimum preference for animal food (Chishti and Peerzada, 1998) as they are mostly dependent on planktons (65-70%) which are the intermediate hosts for *P. kashmirensis*, the rest comprises of aquatic invertebrates. It is generally the amount of intake of intermediate host (which is an invertebrate) that determines the intensity of infection, so the present observation with lower prevalence of infection in *Pomphorhynchus kashmirensis* in its host is a consequence of the minimum quantity of animal food in their diet. Amin (1987) also found a wide host range for *Pomphorhynchus bulbocoli* in Wisconsin fishes, which he attributed to similar feeding habits of the fish and also to the availability of intermediate host in the habitat.

Table. 4: Prevalence of *Pomphorhynchus kashmirensis* in various host species								
Host	**Dal Lake**				**River Jhelum**			***P-Value***
	No. examined	**No. infected**	**Prevalence (%)**	***P-Value***	**No. examined**	**No. infected**	**Prevalence (%)**	
S. niger	84	22	26.19	0.009	66	23	34.85	0.009
S. esocinus	51	7	13.72		44	10	22.73	
S. curvifrons	68	13	19.11		50	19	38.00	
Total	203	42	20.68		160	52	32.5	

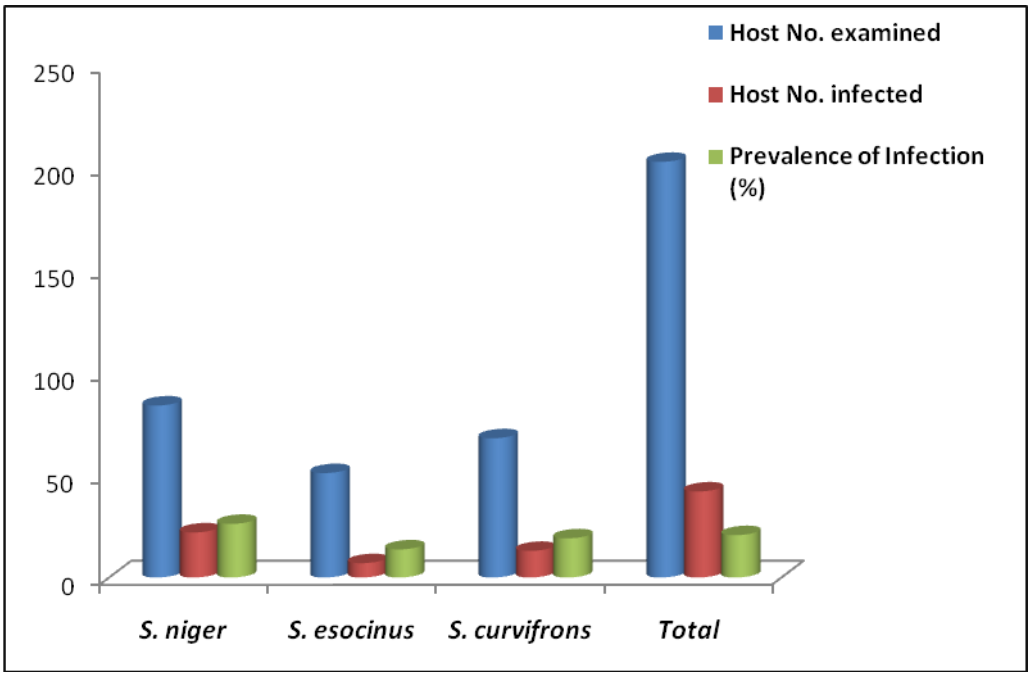


Fig.4: Prevalence of *Pomphorhynchus kashmirensis* in fishes of Dal Lake

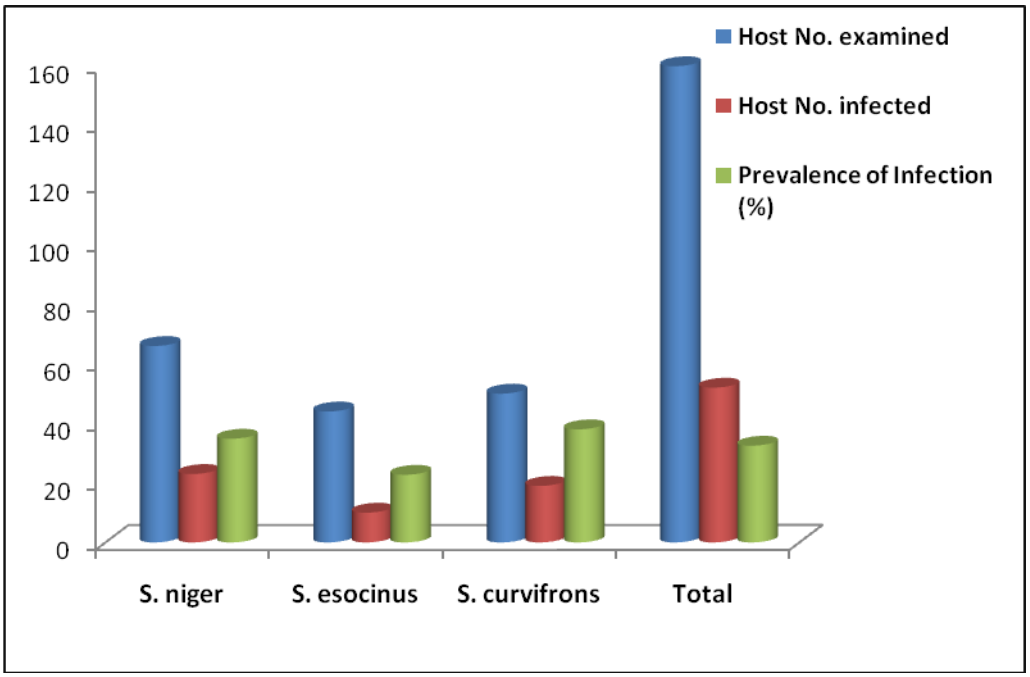


Figure: 5: Prevalence of *Pomphorhynchus kashmirensis* in fishes of River Jhelum

4.1.1. Seasonal prevalence

The data pooled for seasonal estimation of Pomphorhynchus infection revealed definite seasonal prevalence of infection in all the three species of *Schizothorax,* with highest infection in summer and lowest in winter. There was a gradual increase in the prevalence rate from spring to summer and falls down with onset of autumn and least observed prevalence during winter season.

In summer the prevalence was 34.54% (*S. niger*46.34%, *S. curvifrons* 31.11% and *S. esocinus* 30%) and the least prevalence was found during the winter season 8.16% (*S. niger* 14.28 %, *S. curvifrons* 6.89% and *S. esocinus* 9.52%) (Table 5, Fig. 6 and Fig. 7).

Table. 5: Seasonal prevalence of *Pomphorhynchus kashmirensis* in Dal Lake and River Jhelum						
Season	**Host**	**Dal Lake**		**River Jhelum**		***P- Value***
		No. examined	**No. infected (%)**	**No. examined**	**No. infected (%)**	
Spring	*S. niger*	23	5(21.74)	16	5(31.25)	0.05
	S. esocinus	14	2(14.29)	9	2(22.22)	
	S. curvifrons	17	1(5.88)	9	3(33.33)	
Summer	*S. niger*	19	8(42.11)	22	11(50.00)	0.33
	S. esocinus	16	5(31.25)	14	4(28.57)	
	S. curvifrons	20	6(30.00)	15	8(53.33)	
Autumn	*S. niger*	22	6(27.27)	20	6(30.00)	0.11
	S. esocinus	10	2(20.00)	11	3(27.27)	
	S. curvifrons	13	3(23.08)	15	6(40.00)	
Winter	*S. niger*	20	3(15)	8	1(12.50)	0.32
	S. esocinus	11	1(9)	10	1(10.00)	
	S. curvifrons	18	0(0)	11	2(18.18)	
Total		203	42 (20.68)	160	52 (32.5)	

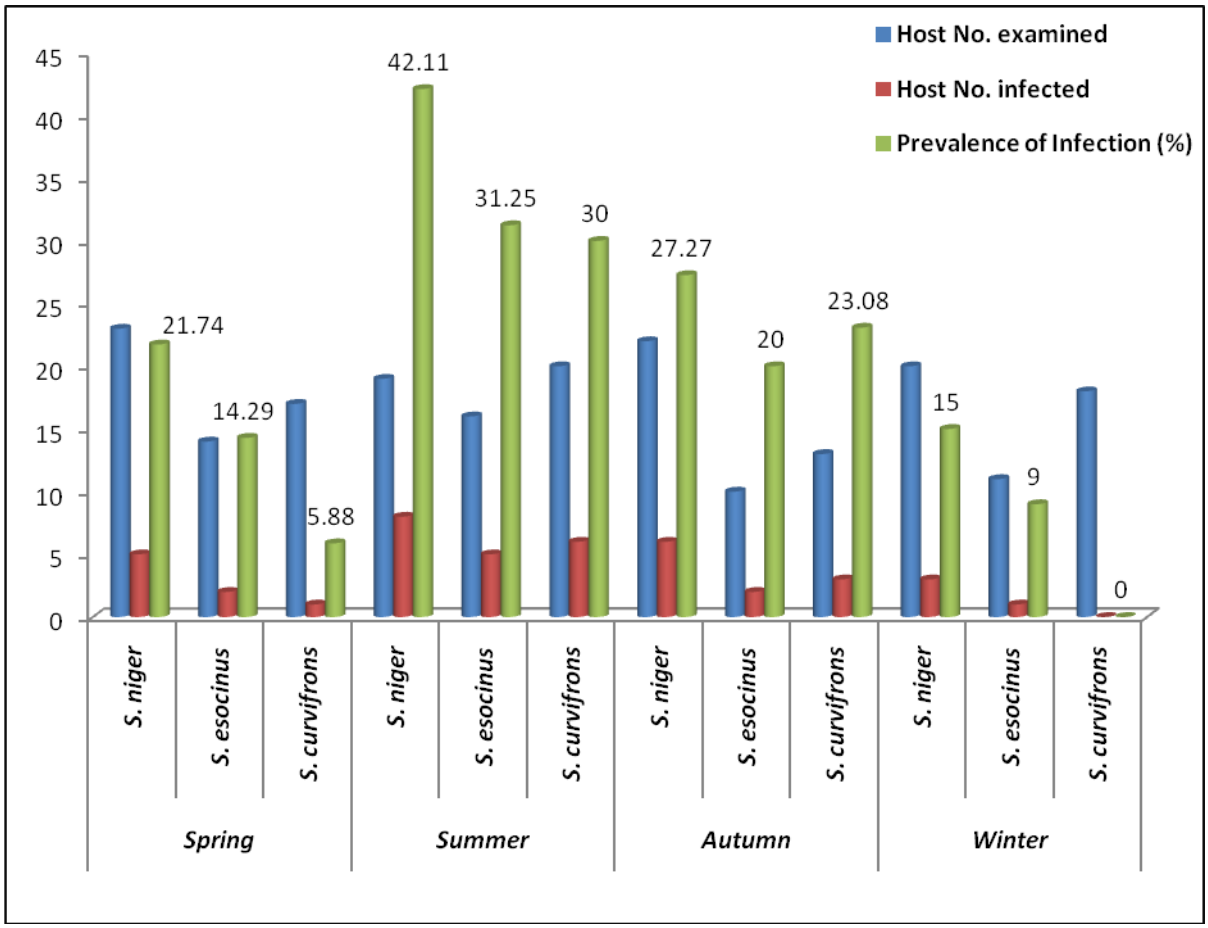


Fig.6: Seasonal prevalence of *Pomphorhynchus kashmirensis* in fishes of Dal Lake

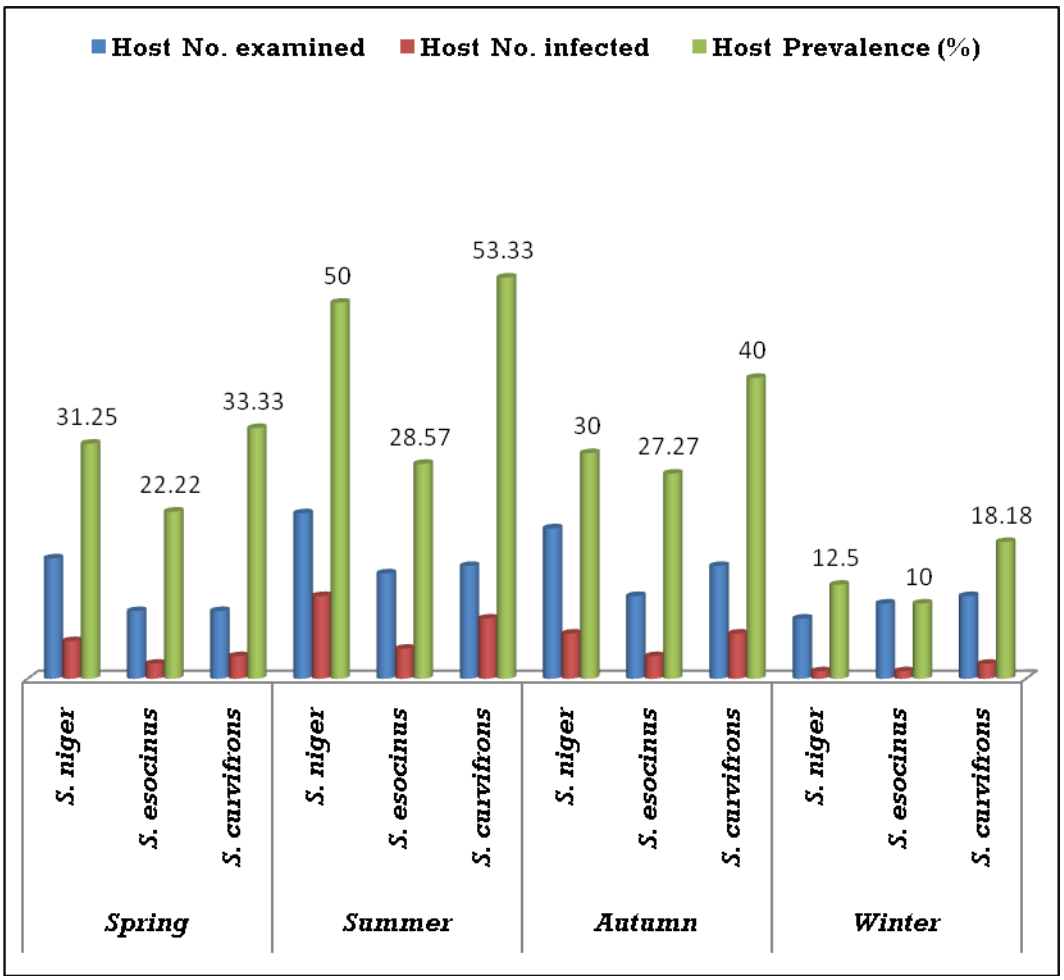


Fig.7: Seasonal prevalence of *Pomphorhynchus kashmirensis* in fishes of River Jhelum

This seasonal variance is quite evident that the highest incidence of *Pomphorhynchus kashmirensis* infection observed during summer and autumn months is attributed to the fact that temperature slowly starts rising above 20 ^{o}C which is favorable temperature for the larval development in the secondary host. This study is in full agreement with Cushing, (1942); Bisset, (1948); Amin, (1975); Andryuk, (1979); Gleason, (1984); Brown, (1989); Khan and Majidah, (1999); Tingbao and Xianghua(2001); Mustafa and Altunel, (2007) and Rubio, *et al.*,(2008). Majidah and Khan (1998) reported the distribution pattern of the helminth populations in different fish hosts, which exhibited a regular seasonal trend and the infrapopulation concentration was relatively greater during summer. This pattern of infection does not conform the study done by various researchers like Chishti and Peerzada (1998), who while working on seasonal occurrence of acanthocephalan infection in fishes of Wular Lake observed that the infection was higher in spring and low from summer in all fish host. Jha,*et al.*,(1992) reported that acanthocephalan *Acanthosentisdalti* showed prevalence of 11.1-76% during different months and having highest incidence in the month of May. Yousuf and Pandit (1996) and Nedeva, *et al.*,(2003) also reported an increase in infection rate in spring and decrease during summer/autumn months. Aloo (2002) however, could not find any seasonality in parasitic infection of fish host.

4.1.2.Gender wise prevalence of *Pomphorhynchus kashmirensis*

After arranging the data, gender wise observations were made which revealed that the sex wise differences were not much prominent but in most cases males 30.30% (*S. niger* 32.50%, *S. curvifrons* 27.65% and *S. esocinus* 28.94%) were found to be more infected than females 27.27% (*S. niger* 27.14%, *S. curvifrons* 36.61% and *S. esocinus* 15.78%) (Table 6, Fig.8).This study is in full agreement with Machado, *et al.* (2000). The influence of sex on the susceptibility of animals to infections could be attributed to genetic predisposition and differential susceptibility owing to hormonal control. It seemed that prevalence of infection by helminth parasites have no sex linked preference which is in accordance to the studies done by Chishti and Peerzada (1998), who reported same infection in the both sexes.

Table.6: Gender wise prevalence of *Pomphorhynchus kashmirensis*

Host	Gender	No. examined	No. Infected	Prevalence (%)	*P-value*
S. niger	**Male**	80	26	32.50	0.013
	Female	70	19	27.14	
S. esocinus	**Male**	38	11	28.94	0.05
	Female	57	9	15.78	
S. curvifrons	**Male**	47	13	27.65	0.10
	Female	71	26	36.61	
Total	**Male**	165	50	30.30	0.01
	Female	198	54	27.27	

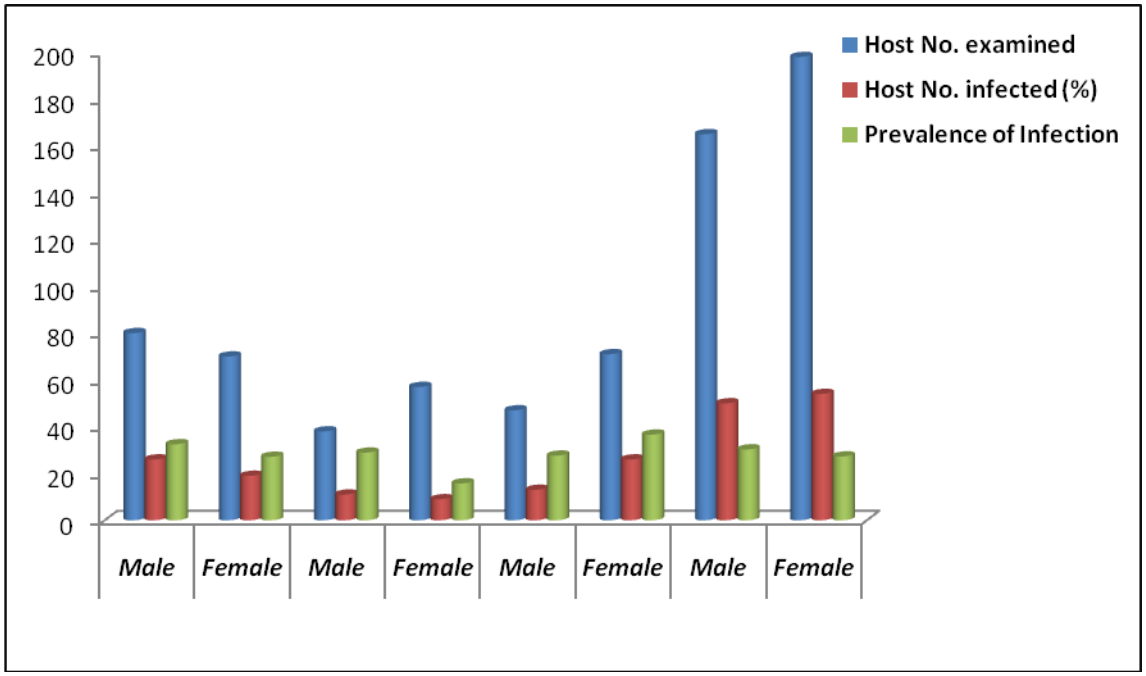


Fig.8: Gender wise prevalence of *Pomphorhynchus kashmirensis*

4.2.Purification and analysis of somatic antigens of *Pomphorhynchus kashmirensis*:

Somatic antigens are the protein moieties that are recognizable by the immune system of an organism or that of a parasite by the immune system of its respective host. A parasitic organism living within a particular host is continuously being challenged by the immune system of the host and the host immune system in turn generates specialized class of proteins called antibodies to eliminate the parasite. These antibodies are highly specific and are directed against the surface or secretory proteins of the parasite. In this particular study, the same property of the host immune system (antibodies) has been utilized to isolate the somatic antigens from *Pomphorhynchus kashmirensis.*

Pomphorhynchus kashmirensis is a parasite worm found in a variety of fish. The sera of the fish can thus be used as a source of antibodies to capture the somatic antigens from *Pomphorhynchus kashmirensis*. For this purpose affinity chromatography is the most appropriate technique to be utilized.

The percentage binding of IgG with CNBr activated Sepharose-4B was 83.74%. Out of a total of 28.0 mg of PSAg loaded in 2 batches on the immunoaffinity column, 0.8374 mg Aff-PSAg was eluted as (E1+E2+E3) with a recovery percentage of 2.97(Photo: 1).

Electrophoretic separation of Aff-PSAg resolved into 5 prominent polypeptides of molecular weight ranging from 29 to 66 kDa (Photo: 2) which is inferred to the presence of 5 or more number of active somatic antigens of *P. kashmirensis*. Hence the isolation of these polypeptides was achieved in pure form. In similar study, low molecular weight polypeptides of Mr<14-33 kDa were predominantly antigenic in *G. crumenifer* (Saifullah *et al.*2000).

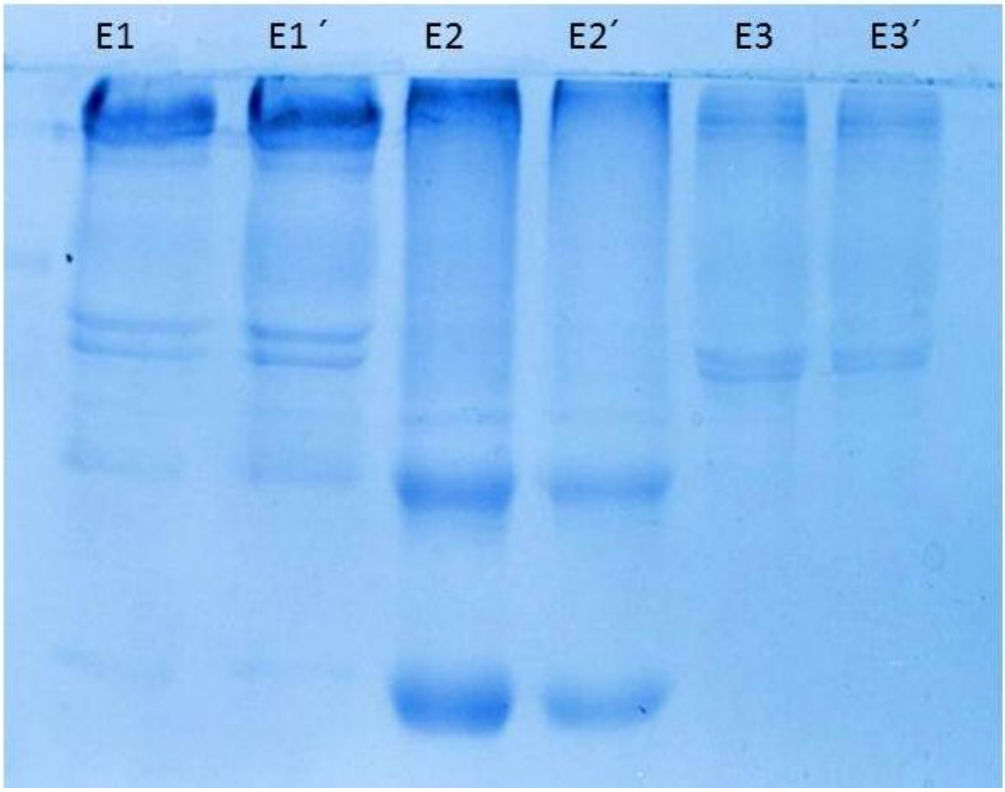


Photo: 1. Analysisof elutes from affinity column showing protein bands of somatic antigens of ***P. kashmirensis*** using 10% SDS-PAGE, MW= molecular weight; E1, E1'= primary elute; E2, E2'secondary elute; E3, E3' final wash of the column.

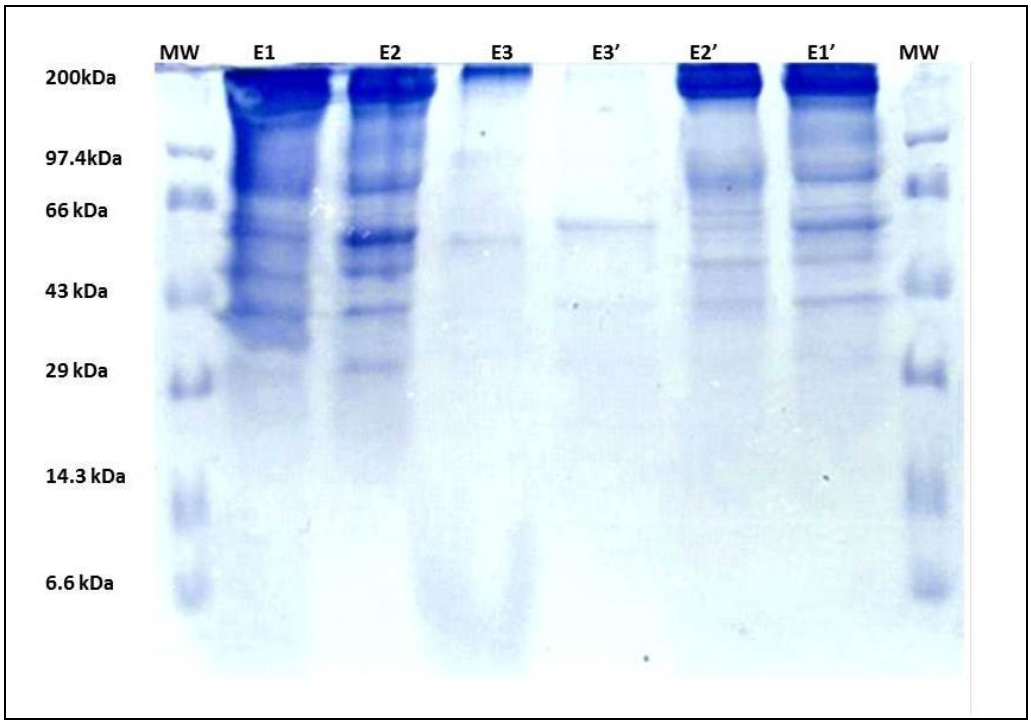
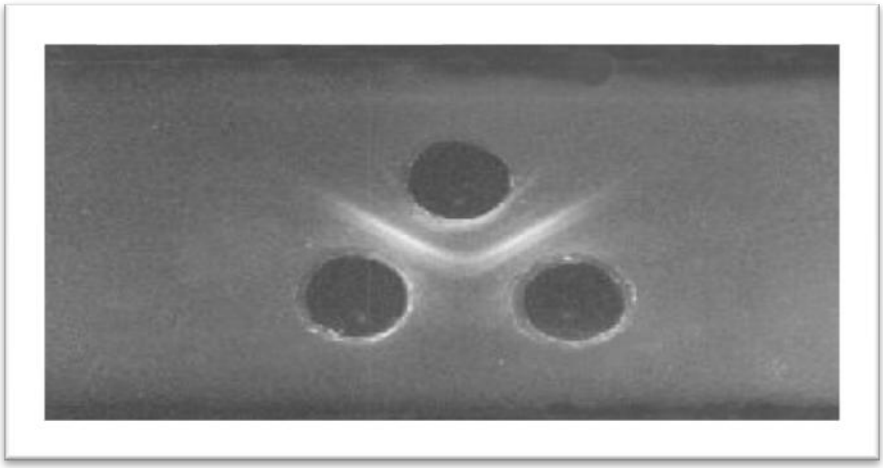


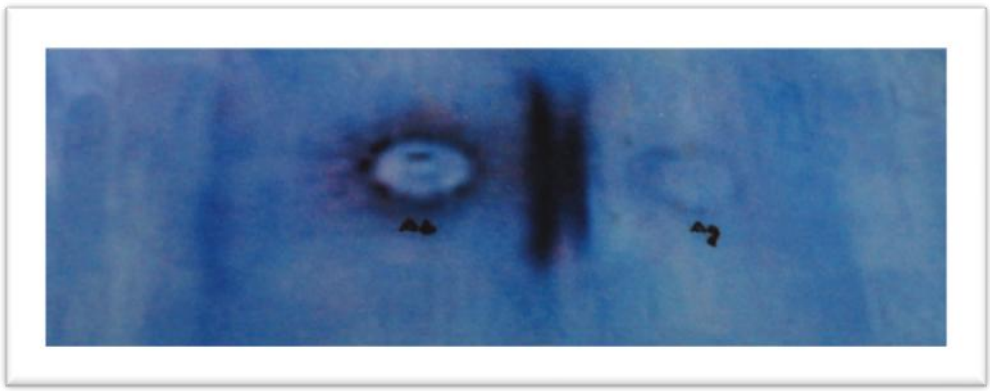
Photo: 2: SDS-PAGE profile of purified somatic antigens of *Pomphorhynchus kashmirensis*

Serodiagnosis by Ouchterlony gel diffusion test:

Somatic antigens (E1+E2+E3) were subjected to Ouchterlony gel diffusion test against hyper immune sera raised in rabbit in order to find the antinegenicity of the proteins. It was performed on agar coated glass slides. Ouchterlony gel diffusion test of somatic antigens showed one precipitation arch against heterogeneous hyper immune sera and many precipitation arches against homogenous hyper immune sera.



(a) Phg. Gel slide showing precipitation line stained with Amido Black Stain



(b) Coomasie blue stained gel showing many (thick) precipitation lines

Photo: 3a & b.Ouchterlony Double Diffusion test against *P. kashmirensis*.

Thus, the results of SDS-PAGE and Ouchterlony gel diffusion test strongly reveals that somatic antigens of *Pomphorhynchus kashmirensis* possess proteins of low molecular weight which mainly ranges between 29 to 66 KDa and are highly immunogenic as revealed by Ouchterlony gel diffusion test. Such observations are in agreement with Megeed (2005). Similarly Ahmad, *et al.*, (2004) proved the antigenic molecules of each fraction were mostly in the low molecular weight range of 14 to 94 kDa. These authors have fractioned the soluble extracts of *Gigantocotyle explanatum,* isolated from the liver of *Bubalusbubalis* on Sephadex G-200 columns. Knopf,*et al.*

(2000)also proved that the humoral immune response of the experimentally infected eels indicates that the antibody response is more likely to be directed against antigens of the adult worms and secondly, the immunoblot analysis revealed the strongest reactions with antigens of adult *Anguillicolacrassus* which were mainly located in the body wall of the adult worms. Coscia and Oreste (2000) while working on the fish nematode *Pseudoterranovadecipiens* antigens using different immunoassays to detect their ability to bind with the fish antibody showed that surface associated proteins have higher binding activity than other kinds of antigens. However, it is not in agreement with Park house, *et al*., (1987) who reported that somatic antigens are poor antigenic in nature. This may be due to the inefficiency of Ouchterlony gel diffusion test. Till date no specific diagnostic polypeptide against *Pomphorhynchus kashmirensis* has been identified and purified. Besides, Affinity chromatography has been shown to be a very effective tool for isolation of candidate diagnostic and vaccine molecules (Sharma, *et al.,* 2001). Pertinently, and as a fact, the results so obtained in the present study may vary because these differences may be due to difference in preparing the antigenic solutions, chemical reagents of different quality and quantity or application procedures (Norouzi, 2007).

In nutshell, it is believed that the somatic antigens derived from the *Pomphorhynchus kashmirensis*can be used as good immunogens and hence can be exploited for mounting the protective immune response in fish. The results of the present study suggest that low molecular weight antigens of *Pomphorhynchus kashmirensis* deserve further investigation as this is the very preliminary study due to time constraint and is first of its kind conducted on *Pomphorhynchus kashmirensis* which is specific to Schizothoracines in Kashmir.

CHAPTER: 5

CONCLUSION

The present research work entitled **"Purification and Characterization of *Pomphorhynchus kashmirensis* Somatic Antigens"** mainly deals with studying the nature of somatic antigens of *Pomphorhynchus kashmirensis*. This endoparasite is commonly found in the lumen of the intestines of fish host. The host specimens were collected from two water bodies viz., the world famous Dal Lake and the River Jhelum. This very parasite was carefully withdrawn from the intestinal lumen and then subjected to various parasitological and immunological studies.

The present study mainly dealt with the following observations summarized as:

- A total of 363 fish specimens of *Schizothorax* species were collected and out of which 203 fishes were collected and examined from Dal Lake and 160 fishes were collected and examined from River Jhelum during the present study.
- On examining 363 fish specimens 94 were found to harbor the *Pomphorhynchus kashmirensis* parasite constituting an overall prevalence of 25.89%.

- Out of 203 specimens examined from the Dal Lake only 42 specimens were found infected with the *Pomphorhynchus kashmirensis* which constitutes the prevalence of 20.68%.

- Similarly out of 160 specimens examined from the River Jhelum only 52 specimens were infected with the *Pomphorhynchus kashmirensis* which constitutes 32.5% prevalence.
- Infection was highest during summer and least during winter.
- In summer the prevalence of infection was 34.54% and in winter season the prevalence infection was 8.16%.
- The results of SDS-PAGE and Ouchterlony gel diffusion test strongly reveal that somatic antigens of *Pomphorhynchus kashmirensis* possess proteins of low molecular weight which mainly ranges between 29 to 66 KDa and are highly immunogenic as revealed by Ouchterlony gel diffusion test and Immunoaffinity Chromatography.

In addition to isolating appropriate antigens for vaccine development, determining their function within the parasite or the host-parasite relationship will give us a better understanding of their importance and help us in the selection of parasite targets. However, functional analysis of parasite antigens in *Pomphorhynchus kashmirensis* itself is hampered by several important drawbacks. Finally, instead of expressing complete worm antigens we can also attempt to design a vaccine that mimics the essential immunogenic epitopes. Identification of these so-called mimotopes can be achieved by further research.

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