DISCUSSION
5. DISCUSSION

Information on structure and character of immunoglobulin of fishes is essential in health management. Adequate knowledge about the characteristics of immunoglobulin provides a strong base for development of diagnostics and vaccines for fishes and also helps to study ontogenic and phylogenetic diversification of antibodies in fishes. Immunoglobulin have been purified and characterized in several commercial fishes such as Japanese eel (Uchida et al., 2000), Snapper (Morrison et al., 2001), Rainbow trout (Sanchez et al., 1993), Sea bass (Bourmaud et al., 1995) and Common carp (Ishikawa et al. 2004).

Purified immunoglobulins of several fishes have been used for developing monoclonal antibodies. MAbs of fish Igs have been applied for different purposes such as to quantify the total Ig in sera at different conditions like healthy, infected, and vaccinated fishes (Wagner et al., 2001; Bobadilla et al., 2004), to detect change in total Ig levels at different seasons, environmental conditions, life stages (Cuesta et al., 2004; Pettersen et al., 2005), to compare the serum antibodies of fishes from different taxas (Al-Harbi et al., 2000; Morrison et al., 2002), to study the heterogeneity in structure of antibodies (Sanchez et al., 1995), to study the ontogeny of antibody production (Breuil et al., 1997), to study the major lymphoid tissues (Pettersen et al., 2000; Jang et al., 2004) and to study the structural changes in fish Ig under different physical, chemical and biological conditions (Magnadottir et al., 1996).

Indian major carps (IMC) form the mainstay of culture fishery in India, supported by a strong traditional knowledge base and scientific inputs in management. Intensification of culture of IMC in recent
years has led to outbreak of several diseases caused by fungal, bacterial and protozoan agents (Kumar et al., 1986 a, b, c; Das and Pal, 1987; Das et al., 1989; Shome et al., 2005). However, serology of IMC in general and immune response and nature of immunoglobulin in particular are not well studied for their use in diagnostics, epidemiological studies or vaccine development for the fish. Moreover in recent immunological studies in Indian Major Carps, the immune response parameters are not including a humoral immunity component or some times contain only a traditional immunological technique like agglutination titre analysis only (Misra et al., 2006). This may be due to the lack of the information regarding their serum Ig or the lack of proper detection technique to quantify the antibody level present in fish.

In the present study rohu serum antibody was purified employing affinity column chromatography using two different columns viz, FMDV recombinant protein linked column and BSA linked column, using sera from fishes immunized with FMDV recombinant protein and BSA respectively to compare characteristics of the purified Ig like proteins. Also a panel of MAb was raised against the purified rohu Igs for the characterization, detection and evaluation of Rohu serum Ig. Using the MAbs an ELISA was standardised for studying the antibody titres at post vaccination and post infection scenario in rohu.

5.1 Antigens for raising rohu antibodies

In the present study FMDV recombinant protein and BSA were selected to raise the antibodies in rohu because of the availability of affinity columns for antibody purification. Immunoglobulins have been purified from fishes immunised with different antigens such as different bacterial preparations (Isbell and Pauley, 1983; Uchida et al.,
2000; Rekha, 2000) particulate antigens like KLH (Ingram and Alexander, 1979) TNP-LPS (Bourmound et al., 1995), DNP-HAS (Grove et al., 2006) Mannan binding protein (Al-Harbi et al., 2000) Protein A (Shin et al., 2006) and Sp A (Estevez et al., 1993) and antibodies like goat IgG (Smith et al., 1993; Pallenzuela et al., 1996; Shin et al., 2006) and Mouse IgG (Jang et al., 2004). Previously in rohu, *Edwardsiella tarda* (Mohanty et al., 1998), foot and mouth virus protein (Srinivas, 2002) and *A. hydrophila* (Rekha, 2000) have been tried to raise immunoglobulin.

For FMDV protein, recombinant plasmid carrying the insert of FMDV (Type A$_{22}$) protein was transformed to *E. coli* cells, induced, expressed and purified in Nickel CL agarose column. The affinity purified FMDV protein was found to be pure on a 12% SDS- PAGE gel with a single 30 KD band. As much as 15 mg of FMDV protein was obtained from 100 ml of induced *E. coli* culture. In earlier studies yield of FMDV recombinant protein was 6 to 12 mg/100 ml culture (Ratish et al., 1999, Srinivas 2002). Earlier Srinivas (2002) also used FMDV recombinant protein for raising Ig in rohu.

BSA has been used as an immunogen for elucidating antibody production in different fishes (Elcombe et al., 1985; Bryant et al., 1999; Swain et al., 2004; Rathore et al., 2006). Although BSA is reported as a poor immunogen (Brayant et al., 1999; Rathore et al., 2006) it is selected in the present study because of the availability of BSA linked affinity column.

An average rohu antibody yield of 2.43 mg and 1.41 mg/ml of sera were obtained from FMDV protein and BSA immunized fishes respectively after purification. Earlier Srinivas (2002) obtained 1.326 mg/ml of rohu Ig with the FMDV protein immunisation. Survey of Ig
levels in different fishes have indicated a range of 0.25 to 23.5 mg/ml sera (Israelsson et al., 1991; Magnadottir and Guomundsdottir, 1992; Uchida et al., 2000; Shelby et al., 2002a).

FMDV protein yielded higher quantity of antibodies than that with BSA. This may be due to the fact that BSA is a less efficient immunogen in fish compared to the FMDV recombinant protein. Earlier Srinivas (2002) also reported that FMDV is a good immunogen in rohu. Brayant et al. (1999) and Rathore et al. (2006) reported poor immunogenicity of BSA in Asian seabass and African catfish respectively.

5.2 Affinity purification of rohu Ig

Rohu serum antibody was purified employing affinity column chromatography using two different columns viz, FMDV recombinant protein linked column and BSA linked column, using sera from fishes immunized with FMDV recombinant protein and BSA respectively. Since both the purification methods are based on antigen-antibody affinity, single step purification was found to be sufficient to get purified antibodies. Srinivas (2002) reported single step purification of rohu Ig by an FMDV column based affinity purification and obtained pure Ig. Rekha (2000) observed that the antibody affinity method gave a better purified protein than gel filtration chromatography. Mohanty et al. (1998) tried to purify rohu Ig by a combination of ammonium sulphate precipitation, gel filtration chromatography and ion exchange chromatography. However, non reducing SDS PAGE analysis of the purified protein showed three bands which indicate impurity in the proteins.
The antigen coated columns could be used for more than three times to purify the Igs with a little decrease in elution efficiency indicating that these single step affinity purification methods are good enough for large scale purification of the Ig. A similar observation was also made by Srinivas (2002).

5.3 Characterisation of affinity purified Ig

5.3.1 Antibody activity

The antibody activity of both the purified Igs were determined using mouse antiserum in immunodot. Previously Magnadottir et al. (1996) employed a mouse anti Atlantic salmon IgM serum to detect antibody activity.

Immunodot revealed that both the purified serum Ig reacted specifically with their corresponding antigens. Earlier Srinivas (2002) determined the reactivity of the FMDV purified rohu Ig by an antigen capture RT- PCR. Rekha (2000) detected the activity of the affinity purified IMC antibodies by an agglutination test using A. hydrophila as the antigen. Swain et al. (2004) confirmed the reactivity of BSA column purified immunoglobulins of Asian catfish using haemagglutination test.

5.3.2 Molecular weight of rohu Ig

Purified Ig were subjected to 3% non-reducing SDS-PAGE to find out their purity and integrity. Anti FMDV Ig did not show any bands in the gel (figure not given) but α BSA Ig showed a single band of the purified protein. Molecular weight of the α BSA Ig was found to be 850 KD similar to the native terameric Ig of many teleosts such as turbot (835 KD, Estevez et al., 1993), African catfish (840 KD, Rathore et al.,
2006), Cod (851 KD, Pilstrom and Petersson, 1991), Atlantic salmon (800 KD, Havarstein et al., 1988), seabass (883 KD, Bourmaud et al., 1995) and tilapia (900 KD, Rajavarthini et al., 2000). It can be seen that with BSA only one form of the Ig (tetramer) was produced and eluted in the affinity purification. Earlier studies on L. rohita (Mohanty et al., 1998; Mohanty, 2001) and Cirrhinus mrigala (Sahoo, 2002) different populations of immunoglobulin molecules such as tetrameric, dimeric and monomeric forms have been reported in the purified sera in non-reducing SDS PAGE. Previously Swain et al. (2006) demonstrated a single 210 KD protein of L. rohita Ig from immunised brood fish serum, egg and larval extracts in non-reducing SDS-PAGE which is believed to be the monomer of the immunoglobulin.

5.3.3 Molecular weight of light and heavy chains of rohu Ig

Purified Ig (both α BSA Ig and α FMDV Ig) were subjected to 10% SDS-PAGE analysis. The α FMDV Ig showed two clear bands of proteins having molecular weights of 59 and 46 KD. Rekha (2000) has reported similar molecular weights (66 and 45 KD) for affinity purified rohu Ig. Srinivas (2002) also reported proteins of molecular weight 63 and 45 KD from affinity purified rohu Ig purified by FMDV column on a 10% SDS- PAGE gel. The slight variations obtained in values in all these studies are probably due to the glycosylation of the Ig, which causes anomalous migration on SDS-PAGE gel (Wilson et al., 1985).

The protein of α FMDV Ig having a molecular weight of 59 KD can be compared with the heavy chain of the Ig of Channel catfish (58 KD, Hall et al., 1973), brown bullhead (50.5 KD, Isbell and Pauley, 1983), rainbow trout (60 KD, Sanchez et al., 1989) and common carp (70 KD, Rombout et al., 1993a). However, the protein having a molecular
weight of 46 KD could not be compared with the light chain of any other fish Ig.

The α BSA Ig had two clear bands of proteins having molecular weights of 85 KD and 23 KD which corresponds to heavy and light chains of the Ig of other species of fishes like tilapia (90KD and 30 KD, Rajavarthini et al., 2000) cod (81 and 27.5 KD, Pilstrom and Petersson, 1991), Trematomus bernacchii (83.5 and 27.5, Scapigliati et al., 1997), turbot (78 and 27, Estevez et al., 1993) and common carp (70KD and 25KD, Rombout et al., 1993a).

5.4 Production of anti rohu Ig Monoclonal Antibodies

Hybridomas producing antibodies were raised against both α BSA Ig and α FMDV Ig separately. The hybridomas were screened for antibody production by Immunodot assay. Immunodot has been used for screening hybridomas earlier by several workers (Anil et al., 2002; Rengpipat et al., 2005; Poulos et al., 2001). Positive hybridomas were minicloned thrice and monoclonal hybridomas were selected for further studies.

5.5 Characterisation of anti rohu Ig Monoclonal Antibodies

5.5.1 Isotypes

A total five monoclonal hybridomas were selected. Three clones (F2D9, F4H9 and B2D1) were of IgG type and two were of IgM type (F4G12 and B4G12). Most of the MAbs reported for various fish Ig were of IgG isotypes except few MAbs of IgM types (Al- Harbi et al., 2000; Sanchez et al., 1993).
MAbs F4G12 and F2D9 were found to be more reactive and others moderately reactive in an Immunodot assay. Anil et al. (2002) also reported highly reactive nature of one MAb in an Immunodot.

5.5.2 Specificity of MAbs

Specificity of each MAb was determined separately by Western blot for α BSA Ig and α FMDV Ig. With α FMDV Ig, except the MAb F4H9 that reacted with the 46 KD protein, all other MAbs reacted with 59 KD protein. Three MAbs, F4G12, F4H9 and F2D9 (raised against Ig purified by FMDV column) were found to be reacting strongly and others reacted weakly. All the MAbs were reacting with the 85 KD protein of α BSA Ig. Three MAbs (B4G12, F4G12 and F2D9) were found reacting strongly and the others weakly with the protein.

All the MAbs were found reacting with the corresponding Ig against which they were developed and also showed cross reactivity with the other Ig. The F4H9 MAbs reacting with the 46 KD protein of α FMDV Ig was also found to react with the 85 KD of α BSA Ig. These results reveal that both α BSA Ig and α FMDV Ig are sharing similar epitopes and thus may be of same origin.

The 85 KD protein of α BSA Ig is considered as the heavy chain of IgM and all the MAbs were reacted with this protein. Earlier studies also indicates that most of the MAbs raised against different fish Igs recognized the heavy chain of Ig (Rombout et al., 1993a; Koumans-van Diepen et al., 1995; Yamaguchi et al., 1996; Lobb and Clem, 1982; Ainsworth et al., 1990; Bang et al., 1995; MacDougal et al., 1995; Killie et al., 1991; Pettersen et al., 1995; Magnadottir et al., 1996; Israelsson et al., 1991; Van der Hejden et al., 1995; Pilstrom and Petersson, 1991; Romestand et al., 1995; Scapigliati et al., 1996;
Dos santos et al., 1997; Thuvander et al., 1990; Sanchez et al., 1995; Estevez et al., 1994; Adkison et al., 1996; Jang et al., 2004; Shin et al., 2006; Al- Harbi et al., 2000; Morrison et al., 2002; Shelby et al., 2003).

5.6 Epitope analysis of fish Immunoglobulin by anti rohu Ig Monoclonal Antibodies

5.6.1 Reaction of MAbs with serum and Mucus Ig

The MAb F4G12 was found to be reacting with both sera and mucus of rohu. Similar observations have been reported by others where anti- serum Ig MAb reacted with serum and mucus Ig (Rombout et al., 1993a; Cain et al., 2000;). Such MAbs have been employed to differentiate serum and mucosal Ig (Cain et al., 2000; Hatten et al., 2001; Bromages et al., 2006 Drennan et al., 2007) and their quantification under different infections (Zilberg and Klesius 1997; Klesius, 1990; Fuda et al., 1991; Estavez et al., 1995; Sanchez et al., 1993; Joosten et al., 1997). Zilberg and Klesius (1997) utilised a MAb to channel catfish Ig for quantification of Ig in the serum and mucus at different ages following infection with Edwardsiella ictaluri. Drennan et al. (2007) employed a white sturgeon anti Ig MAb for the characterization of both serum and mucosal Ig against White sturgeon iridovirus.

5.6.2 Cross reactivity of MAbs with different fish sera

Most of the cross reactivity studies of anti – fish Ig MAbs with the serum of other fishes were either based on Western blot analysis (Adkison et al., 1996; Morrison et al., 2002; Al- Harbi et al., 2000; Mac Dougal et al., 1995) or ELISA (Scapigliati et al., 1996; Romestand et al., 1995; Thuvander et al., 1990; Sanchez et al.,
In the present study an Immunodot assay was employed for analyzing the reaction of the panel of three MAbs with sera from a wide range of fishes.

The reaction of MAb F4G12 with sera of cyprinid fishes and F2D9 with all cyprinids except common carp reveals that they recognize an epitope that are common to cyprinid Igs. B4G12 reacted with all fish sera and thus its target epitope may be common for all tested fish Igs. Cross reactivity of an anti fish IgM MAb with other fish Igs have been reported by a number of workers (Thuvander et al., 1990; Adkison et al., 1996; Morrison et al., 2002; Israelson et al., 1991; Al-Harbi et al., 2000; Mac Dougal et al., 1995). Miyadai et al. (2004) reported the cross reactivity of MAbs for Takifugu rubripes with other species of Takifugu species. Sanchez et al. (1993) reported cross reactivity of a panel of 12 anti rainbow trout Ig MAbs with different trout and salmon species. The anti southern bluefin tuna Ig MAb was reacting not only with other tuna species but also light cross reaction with barramundi and striped trumpeter (Watts et al., 2001). It has also been demonstrated that there is a significant homology between the amino acid sequences of IgM µ chain from very diverse species (Bengten et al., 1991, Andersson and Matsunaga 1993; Lee et al., 1993). Cross reactivity of the MAbs with different fish Igs can be utilized for the detection of related Igs by immunological assays. Cross reactivity of anti trout Ig Mabs with Atlantic salmon Ig has been utilized to detect Atlantic salmon Ig by ELISA (O'Dowd et al., 1999; Bricknell et al., 1999).

5.7 Detection of rohu Ig by MAb based ELISA

Immunoglobulins in fish sera can be detected by several techniques such as, immunodot, westernblot, agglutination titre and
ELISA among which ELISA and agglutination titres are mostly applied as quantitative assays. The sensitivity of ELISA was found to be six times higher than the agglutination method for detection of antibodies in serum of Japanese flounder (Furuta et al., 1995). ELISA is believed to be the best method to measure specific antibody titre in fish (Morrison and Nowak, 2002) with high sensitivity and specificity (Dixon et al., 1994). Many workers have employed ELISA for the detection of Ig in fish (Thuvander et al., 1990; Iida et al., 1991; Stromsheim et al., 1994; Romestand et al., 1995; Estevez et al., 1995; MacDougal et al., 1995; Van der Heijden et al., 1995; Leiro et al., 1996; Scapigliati et al., 1996; Dos santos et al., 1997; Coeurdacier et al., 1997; Wagner et al., 2001; Shelby et al., 2002b; Bobadilla et al., 2004).

In the present study an ELISA was standardized to detect antigen specific immunoglobulins in rohu. Development of serum Ig in rohu was evaluated through an experimental infection study and a vaccination study. A. hydrophila was selected as the antigen because it was reported to be the most precarious pathogen to rohu (Gopalakrishnan, 1961; Shome et al. 2005). MAb F2D9 was used in the assay because of its higher sensitivity and specificity.

During the infection studies mortality was observed among fishes. Clinical signs of fishes and the reisolation of A. hydrophila from blood revealed that the fishes died due to A. hydrophila infection. Similar clinical signs were reported in rohu by Nayak et al. (1999).

Both vaccinated fish sera and infected fish sera were subjected to an antibody capture ELISA to demonstrate the ability of the MAbs to determine antigen specific antibodies. From the ELISA titration curves it is clear that there is a clear demarcation in the antibody titration
between control, immunized and infected sera. There is a decline in antibody titre from the 5\textsuperscript{th} dpi sera to the 15\textsuperscript{th} dpi sera and the infected sera shown the titre values in between. As the dilution of the test sera increased, absorbance decreased linearly ranging from the 1:100 to 1600. Similar results were reported by many workers for different anti fish Ig MAbs employing different antigens. Van der Heijden \textit{et al.} (1995) demonstrated the suitability of the anti European eel Ig MAbs to detect antigen specific antibodies using similar ELISA titration curves. Zilberg and Klesius (1997) demonstrated the antigen specificity of catfish immunoglobulin employing an anti catfish IgM MAb based ELISA. ELISA has been developed to quantify specific antibodies against different antigens (Bobadilla \textit{et al.}, 2004; Wagner \textit{et al.}, 2001; Shelby \textit{et al.}, 2002b; Estevez \textit{et al.}, 1995; Thuvander \textit{et al.}, 1990).

5.8 Overall characteristics of rohu Immunoglobulins

In brief, SDS PAGE, non-reducing SDS-PAGE and Western blot results indicate that BSA column purified protein is the tetrameric Ig of rohu. Even though both BSA and FMDV purified proteins shows antibody activity, the Native (850 KD), heavy and light chain (85 and 23 KD respectively) molecular weights of BSA column purified protein only could be compared with other fishes. Western blot results reveals that both the purified proteins are sharing epitopes indicating that the proteins may be of same origin with Ig characters. FMDV based column purified protein may be a degraded part of the Ig or a halfmere of Ig. Previous reports suggest that the degradation of the Ig can be due to the fluctuation in storage temperature (Acton \textit{et al.}, 1971) enzymatic action (Van Ginkel \textit{et al.}, 1991; Magnadottir \textit{et al.}, 1996) or chemical denaturation (Kaattari \textit{et al.}, 1998; Watts \textit{et al.},
Since urea is used in purification of sera in FMDV column, trace of urea present in the column might have denatured the eluted Ig. Similar chemical degradations were also reported by several workers. Upon denaturation with SDS, a portion of the tetrameric teleost IgM has been shown to split into mono, di and trimers, depending on the species (Glynn and Pulsoford, 1990; Lobb and Clem, 1981; Whittington, 1993; Kobayashi et al., 1984; Bromage et al., 2004; Pucci et al., 2003).

Since the molecular weight of purified Igs are low, the α FMDV Ig can also presumed to be a halfmers of the tetrameric IgM. Number of low molecular weight Igs (generally monomers) have been isolated from various fish species (Watts et al., 2001). Previous studies on fish Igs suggests that low molecular weight Igs can be either simple monomer (Clem and Mclean, 1975; Warr, 1983; Whittington, 1993) dimer (Glynn and Pulsford, 1990) or a structurally distinct low molecular weight Ig (Lobb and Clem, 1981). A number of teleost fishes have the ability to produce heterogeneous mixture of IgM polymers by simple variation in the degree of disulphide polymerization of monomer or even halfmer subunit (Lobb and Clem, 1981, Bromage et al., 2004). This heterogeneity in the basic structure refered to as redox forms has important implication for macromolecular assembly process and, potentially, for the generation of teleost Ig functional diversity (Kaattari et al., 1998).