Chapter 5: Discussion..

5. Discussion

The principle objective of the modern aquaculture practices involves income generation along with health improvement of the population. However, diseases are major threat towards successful aquaculture. Aquatic animal diseases cause extensive loss to aquaculture based Industries and the losses had exceeded six US billion $ per annum (World Bank 2014). Antimicrobials and vaccines development had provided a creditable progress in the prevention, control and even suppression of contagious diseases. The most important causes of global disease are due to infectious diseases in aquaculture industries with high rate of morbidity and mortality.

The aim of the present study includes the molecular identification and pathogenecity study of the bacterium responsible for infection and mortality in *L. rohita*. In the K. pneumoniae infected fish samples, the fishes were showing reddish lesions and hemmorhages on their body surface. Bacterial identification, biochemical tests, 16S rRNA gene sequence analysis and challenge studies in *L. rohita* have been performed to confirm infection *caused by K. Pneumoniae.* There were different standardized microbiological culture techniques used for the isolation and identification of pathogen. Gram staining revealed the isolated strains as gram negative isolates. Ledeboer et al., 2015; Saurabh et al., 2018 have also reported K. pneumoniae as a gram negative bacteria. Earlier media like MacConkey agar, EMB agar, LB media were used for the isolation of *K. pneumoniae* (Bruce et al., 1981; Maal et al., 2014). However, the reported media were not able to segregate K. pneumoniae from other microbes. So presently a new media *Klebsiella* selective agar supplemented with specific antibiotic was used for the segregation and successful isolation of *K. pneumoniae*.

The major biochemical tests like indole, urease, voges- proskauer and methyl red are required for differentiation of pathogenic *K. pneumoniae*. Biochemical test result of all the isolates were found similar with reported work of different researchers (Gopi et al., 2016; Takyi et al., 2012; Diana and Manjulatha, 2012). All isolates were found positive for urease. Urease helps bacteria to flourish under harsh acidic condition. It is also helpful for bacterial colonization, survivality and pathogenecity (Mobley and Warren, 1996; Maroncle et al., 2006; Burne and Chen, 2000). Based on 16S rRNA sequence analysis of first phylogenetic tree, it revealed that K1 isolate was having a close evolutionary relationship with *K. pneumoniae*. They were sharing the same node with highest bootstrap value. The second phylogenetic tree revealed that the isolate from Nadia (K10) and North 24 Parganas (K5) were very close to each other and were showing 100% homology. In spite of different geographical location, their closeness reveals conserved 16S rRNA region throughout the evolutionary process. Also there has been a transfer of strain from one location to other. Throughout the bacterial genome, 16S rRNA gene is extensively distributed. It is widely used for the identification of bacteria (Woo et al., 2009). It is a subunit of 30S ribosome and having a size of around 1500 bp. Due to its high stability it is being globally used as a molecular marker, taxanomic identification and also for studying the evolutionary relationship (Singh et al., 2011). Due to specificity and accuracy of 16S rRNA gene, According to it is highly beneficial for the molecular identification of the bacterial contaminants (Rhoads et al., 2012; Gee et al., 2003; Rompreet al., 2002). Gopi et al., 2016 have used 16S rRNA gene sequence analysis for the identification of *K. pneumoniae* from infected *A. nigripes.*

In case of any infections or diseases, the accurate microbial identification of the pathogens at species and strain level is very much important. So PCR ribotying

had found to be a promising technique for studying the presence or absence of any polymorphism at the ISRs (García-Martínez et al., 1999). Lopes et al., 2007 had used specific primers to differentiate *K. pneumoniae, K. oxytoca* and *E. aerogenes*. *GyrA* and *parC* gene PCR were unable to distinguish *Klebsiella pneumoniae* at subspecies level. However, PCR ribotyping had differentiated three subspecies of *K. pneumoniae* (Brisse and Verhoef 2001). Presently, a consistent and conserved ribotype pattern was observed for all the isolates. Thus suggesting thet PCR ribotyping can be used as an alternative tool in spite of traditional biochemical tests for microbial identification upto species level. In present day aquaculture practices, fish farmers are using various kinds of antibiotics without having the proper information about the fish pathogen. However, continuous antibiotic use in aquaculture ponds is becoming an alarming threat. Pathogens are getting resistant to multiple antibiotics (Ventola, 2015). Penicillin group antibiotics like tetracycline, imipenem, amoxycilin, dicloxacillin, ampicilin and piperacillin are commonly used for the treatment of *K. pneumoniae*. According to Williamson et al., 1986, synthesis of cell wall of bacteria can be inhibited by penicillin group of β- lactum antibiotics. Presently, all isolates were resistant against maximum antibiotics having β-lactum. Thus the isolates were producing ESBL (extended-spectrum β-lactamase) enzyme which is helping the isolates to resist against those antibiotics. From Europe Knothe et al., 1983, have first time reported the *K. pneumoniae* ESBL strain. It is difficult to treat infections due to ESBL-producing bacteria as they are co- resistant in nature and there are limitations for choosing antibiotics for treatments. Furthermore, present isolates were found resistant against many third and fourth generation antibiotics *viz.* Ceftazidime, Cefixime, Cefipime. Multiple antibiotic resistance of bacteria is mainly due to the existence of plasmids. Those plasmids can have one or more antibiotic resistance

genes (Sandhu et al., 2016). In the present study the MAR index have been found to be more than 0.2. Thus the MAR index results were showing origination of the strains from an environment where antibiotics are frequently being used. Hemolysis of blood by gram negative bacterial isolates shows the virulence and pathogenic potentiality of the isolate (Barrett and Blake, 1981; Sharma and Gupta, 2014). Albses, 1989 have first time reported the hemolysin activity of *K. pneumoniae* on blood agar having rabbit blood. In the present study all the isolates were found to be positive for β-hemolysin on blood agar plates. In present research work, microbial property of RBCs hemaaglutination was carried out to evaluate the virulent property of the strains. The finding of the present study was in agreement of the reports submitted by Gundogan and Yakar, 2007; El-Sukhon, 2003; Younis et al., 2016. From the liquid haemolysin assay it can be suggested that *K. pneumoniae* can cause haemolysis within 12 hpi.

In the present research work, in vivo challenge experiments were conducted to determine the lethal dose of *K. pneumoniae* required for 50% mortality in *L. rohita*. The calculated LD_{50} was found as 1.05×10^6 CFU/ fish. The estimated LD_{50} value will be useful for studying different immune related experimental studies. The external clinical signs of the artificially infected fishes were similar as of the infected fishes collected from aquaculture farms. From the internal organs and blood of the moribund fishes, bacteria was reisolated and reconfirmed as *K. pneumoniae* by growing them on *Klebsiella* specific agar plates and also by biochemical characterization. Histopathologically in artificially infected *L. rohita* kidney tissue, focal necrosis, vacuolation and structure alterations in glomerulus were observed. Abraham et al., 2015 had also reported the similar kinds of alteration in kidney after artificial infection of *C. gariepinus* with *E. tarda*. Necrosis and glomerular structural changes were observed by Ventura and Grizzle, 1988 after challenging *Ictalurus*

punctatus with *A. hydrophilla*. Gobinath and Ramanibai, 2014 had also observed tubular vacuolation and necrosis in the kidney tissue after challenging *L. rohita* fingerlings with *V. cholera*. However, the histology of the liver tissue revealed necrosis, melanomacrophages center formations, vacuolation and disrupted hepatocytes. Gobinath and Ramanibai, 2014 had also observed vacuolations, disrupted hepatocytes and necrosis after challenging *L. rohita* fingerlings with *V. cholera*. Ramkumar et al., 2014 have reported necrosis of the hepatocytes after artificially infection of *Providencia vermicola* in *L.rohita.* Similar kind of alterations was observed by Gopi et al., 2016 in the liver tissue of *A. nigripes* after *K. pneumoniae* infection. Ly et al., 2009 have observed necrosis of liver after *E. ictaluri* infection in *Pangasianodon hypophthalmus.*

Hypermucoviscous phenotype is considered as an important virulent property of *K. pneumoniae* strains (Sharma et al., 2015). In the 1980s, hypermucoviscous strain of *K. pneumoniae* was first reported from Taiwan (Fung et al., 2002). It was showing characteristics as similar to the classical strains reported earlier. However, it was also hypermucoviscous in nature. Thus making it more pathogenic and hypervirulent (Catalán-Nájera et al., 2017). Later, there were many reports of hypermucoviscous *K. pneumoniae* from Korea, Japan, Vietnam, North and South America, Australia, South Africa, Caribbean and Europe (Shon et al., 2013). There is a difference between mucoid and hypermucoid colonies. The hypermucoid colonies grown on agar plates while stretched with a loop will produces a viscous string of about ≥ 5 mm in length. The present research outcome was found familiar with the work of preceding researcher (Yu et al., 2006; Shon et al., 2013; Fang et al., 2004). An extra capsular polysaccharide mucoviscous web is produced by virulent hypermucuviscous trait. It makes the bacteria resistant to phagocytosis by the host neutrophils (Gupta et al.,

2003). *K. pneumoniae* produces many important factors are produced by that are found to be important for virulence of this bacterium.

Till date, many workers have reported about the virulent genes of *Klebsiella pneumoniae* like *fimH, fimA, mrkA, ecpA, urea, entB, uge, wabG, magA, rmpA, kfuB, allS*. Among this virulent factor, pili are essential for primary colonization on the host and also for capsular polysachride production. This capsular polysaccharide protects the bacteria from bactericidal action of serum, phagocytises and impairs host cell defence (Álvarez et al., 2000). The essential step for development of any infection by a bacterium is by adhesion on the host mucosal surface and in case of gram negative bacteria this property is achieved by filamentous organelles known as fimbriae. These organelles are usually present on the bacterial cell surface (Struve et al., 2008). *K. pneumoniae* have the capability to produce two type of fimbrial adhesion .The first type is known as mannose sensitive Type 1 pili. Type 1 pili is mainly made up of major fimbrial subunit (*fim A*) and minor (*fim H*). The minor subunit identifies the mannose-containing glyco- proteins present on the host tissue thus allowing the bacteria to target and attach on the host tissue (De Vries et al., 1996; Martinez et al., 2000). The second type is known as type 3 pili. In type 3, the major pilus subunit is (*MrkA*) and a minor adhesion subunit is (*mrkD*) (Alcántar-Curiel et al., 2013; Schroll et al., 2010). They are characterized by their capability of agglutination human erythrocytes (MR/ K agglutination) (Duguid, 1959; Clegg et al., 1994). Watnick and Kolter, 2000, have reported that biofilm formation by bacteria have played a promising role in development of diseases. A biofilm formation is a complex process mediated by bacteria (O'toole and Kolter, 1998). Langstraat et al., 2001 have reported that the major type 3 fimbrial subunit (*MrkA*) is mainly responsible for the biofilm formation. Presently the isolates were found to be positive for both major and minor

fimbrial subunits. *ecp* gene had been reported to play a key role for biofilm development and also helps the bacteria to discriminate between the host cells (Schroll et al., 2010). Alcántar-Curiel et al., 2013 have also reported the role of ECP for micro colonies development on epithelial cells and also involvement in stable biofilm formation.ECP gene was firstly reported in *E. coli* however, it was reported later in *C. rodentium, S. boydii* and *K. pneumoniae*. All the strains of the present study have been found to be positive for *ecp* gene and finding were found to be similar with the reports of Alcántar-Curiel et al., 2013; Shon et al., 2013.

When a bacterium enters in the small intestine, it had to encounter many different factors like alterations in pH, bile and different nitrogenous waste. However, for infection bacteria must be able to adapt with the challenging environment (Maroncle et al., 2006). *K. pneumoniae* have been reported to utilize different compounds like histidine, arginine, proline and urea as the sole carbon source (Collins et al., 1993). Presently all the isolates were found to be positive for urease activity both biochemically and also by PCR amplification (Fig. 25). Burne and chen, 2000 had also reported that urease released by *K. pneumoniae* had caused tissue damage and also helped the pathogen to stay for a long period of time. According to Shankar-Sinha et al., 2004 there are few *K. pneumoniae* strains that posses both O (smooth lipopolysaccharide) and K (capsule polysaccharide) antigen. *wabG* and *uge* genes plays a vital role for the expression of these antigens and also for the virulence. *WabG* is mainly involved in biosynthesis of the lipopolysaccharide and the Uge gene is usually present in the outer lipopolysaccharide (Aljanaby, 2017). The outer lipopolysaccharide is the most important molecule responsible for the immunity of the bacterial species (Izquierdo et al., 2003). Regué et al., 2003 have reported that mutation in *uge* gene will led to generation of O-ve: K -ve isolates. According to a

report by Regué et al., 2004, *K. pneumoniae* lacking *uge* gene were less virulent towards the laboratory animals. According to Bach et al., 2000, few *Klebsiella pneumoniae* strains have the ability to produce a huge amount of capsular polysaccharide. It protects the bacteria from phagogocytosis and it is also responsible for the development of mucoid property of the bacterial isolates. Yeh et al., 2007 have reported that *rmpA, uge* and *wabG are mainly responsible for the* development of hypermucoviscosity phenotype and capsular biosynthesis. In the present study the isolates were found to be negative for *rmpA* gene. However, it was found to be positive for *uge* and *wabG* gene. Iron is one of the important factors for the bacterial development inside the host and it also provides the nutrients required for bacterial growth and survival (Aljanaby, 2017). Kannahi and Senbagam, 2014 have reported that *K. pneumoniae* posses few genes like *entB* (enterobactin), *kfu* (iron uptake) and *ybt* (yersiniabactin) genes for the extraction of iron from the host carrier proteins like heme or transferrin. Enterobactin have the ability to isolate iron from such environment where the concentration of iron is very low. It also helps the bacteria to extract iron from air (Sebbane et al., 2010). Presently, all the isolates were found positive for *entB* gene. May and Okabe, 2011 have reported that enterobactin expression have induced biofilm formation. Lai et al., 2001 have reported that enterobactin genes were activated when bacterial infections had occurred.

The present study revealed the alteration in the innate immune system and expression of some *L. rohita* immune related genes infected with *K. pneumoniae* at different time interval. Due to heterothermic nature and limitation in antibody repository of fin and shellfishes, the adaptive immune system of those organisms have some limitation. So they are mainly dependent on their innate immune system for combating against any invading pathogen (Dunier and Siwicki 1994; Whyte, 2007).

The innate immune system recognizes and responds to pathogens in a nonspecific way (Srivastava and Pandey, 2015). When the host encounters foreign polysaccharide particles (PAMPs), the innate immune system gets initiated. Once the PAMPs got recognized, then in turn pattern recognition proteins (PRPs) will bind to it which then sequentially activates the process of phagocytosis and the prophenoloxidase (proPO) activating system (Janeway and Medzhitov, 2002; Cerenius et al, 2008). When an infection occurs, primarily reactive oxygen species (superoxide radical and hydrogen peroxide) are released by cells. Once the phagocytic cell membrane gets stimulated, then in turn it will intiate the production of reactive oxygen species (ROS) which is being reported to be toxic against bacterial pathogen (Sharp and Secombes, 1993; Secombes and Fletcher, 1992; Secombes, 1996). Reactive oxygen species had been reported as an important parameter for fighting against pathogen. According to a report by Sharp and Secombes, 1993 inactivation of ROS by enzymes have no effect on bacterial killing by macrophage. H_2O_2 and free radicals are required by ROS for its function. During the challenge study, the superoxide production as estimated by NBT assay was found or be significantly higher in the infected group on comparison with the control group (Fig. 30a). Similar kind of observations have been reported when tilapia were injected with *Aeromonas hydrophilla*. In a report by Das et al., 2011, increased level of superoxide production has been observed in the infected fishes. In host defence against many bacterial, viral and fungal infections, neutrophils play an important role. An elevated role of neutrophils can be useful for evaluating the health of the host population (Densen and Madell, 1990). During inflammation an enhanced level of neutrophils and macrophages in the blood can be observed (Srivastava and Pandey, 2015). In the present study an elevated myeloperoxidase activity of serum was observed in the artificially infected fishes (Fig. 30b). Similar kind of result was

demonstrated by Das et al., 2011. The elevated activities of superoxide and myeloperoxidase indicate the initial inflammatory response of fishes in response to the invading pathogen. Fish plasma cell carries various protease inhibitors mainly α-1, α -2 antiprotease and α -2 macroglobulin (a-2 M) (Ellis, 1987). According to Rao and Chakrabarti, 2004, this antiprotease restricts pathogen entry and the production of extracellular enzymes. Few bacterial species produces proteolytic enzymes that have the ability to use host protein as an amino acid source. Sometimes during infection and immunization, the activity of these antiproteases remained unaffected (Magnadottir, 2006). In our study, both the anti- protease and α-2 M activity was significantly decreased in the *K. pneumoniae* challenged rohu (Fig. 30 c & d). Similarly (Das et al., 2011) had observed no significant difference in antiprotease activity after injecting *P. sarana* with *A. hydrophila*. From there it was understood that proteolysis cannot be performed, if the bait' region can't be cleaved by protease (Das et al., 2011). From our result it can also be hypothesized that *K. pneumoniae* can produce huge amounts of proteases so that the host antiproteases enzymes are unable to fight against it. NAG and NAM are the main component of the bacterial peptidoglycan layer. Lysozyme enzyme hydrolyses peptidoglycan layer of bacterial cell walls. The presence of lysozyme had been observed in mucus, leucocytes rich tissue and serum of the fishes (Ellis, 1999). In the challenged fishes lysozyme activity of the serum was found to be significantly higher than the control group till the end of the exposure (Fig. 30e). Similar kind of lysozyme expression was reported in sheat fish, Japanese flounder and rohu (Caruso et al., 2002; Hikima et al., 1997; Mohanty and Sahoo, 2010).

Complement system is the key component of innate immune system of the host (Gasque, 2004).In immune response, Complement system is found to be the first

line of immune defence system. It is the principal component of the host immune system (Gasque, 2004). The significant function of complement system includes direct killing of pathogens, production of opsonin molecules and maintaining homeostasis. Three different pathways are involved in the activation of complement system *viz*. the classical pathway (CP), alternative pathway (AP) and lectin pathway (LP) (Gasque, 2004). C3 component plays an important role for complement system activation (Del Rio-Tsonis et al., 1998). The most important role of C3 in complement pathway system includes membrane attack complex (MAC) formation. Thus it will destroy the invading pathogens. Das et al., 2011 had reported that during infection C3 is the most important antimicrobial weapon and defence mechanism of teleosts opsonisation. In the present study, it was found that expression of C3 in the liver was induced gradually in *L. rohita* from 12 hours to 24 hours following bacterial challenge; however the expression significantly lowers in 36 and 48 hours probably due to lowering of infection load (Fig. 31A). Moreover expression of C3 was found to be higher in 72 hours to maintain tissue homeostasis. Further, the gene expression of C3 was measured in kidney and muscle as well. Interestingly in kidney tissue late onset of bacterial infection was evident from the gradual increase of C3 gene expression in later stages of study where 72 hours showed significant elevation of complement protein. However in muscle C3 expression level remain unaltered in all the treated groups. Dash et al., 2017 have also reported that after challenging *Labeo rohita* with outer membrane protein of *A. hydrophilla*, an increased C3 gene expression at the early stages of infection was observed. According to their finding it can be concluded that after injection of the pathogen the complement system of the fishes had got activated and in turn it had tried to kill the pathogen through opsonisation.

Among the cytokines, Interleukin-6 is a pleiotropic nature of cytokines and involved in innate immune responses, adaptive immune responses and various physiological processes (Varela et al., 2012; Naka, et al., 2002). The IL-6 family have a very important role in hematopoiesis (Wang and Secombes, 2009). It had been described as a proinflammatory cytokine due to its anti-inflammatory and immune suppressor properties. Various cell types like macrophages, glial cells, fibroblasts, keratinocytes and endothelial cells are responsible for production of IL-6 (Spooren et al., 2011). IL-6 plays an important role in re-establishing normal liver function after a subsequent liver injury (Taub, 2004). According to Gauldie et al., 1992, when an injury occurs, IL-6 molecules were being generated by the immune cells. Those IL-6 molecules signal the hepatocytes for the production of various acute phase proteins. From the result of the present experiment an anticipation can be made that when the normal functioning liver was exposed to *K. pneumoniae* infection, it had led to production of IL-6 molecule. Earlier we have shown that complement component C3 production in bacterial-stimulated hepatocytes. The combined data suggests that bacterial infection induces hepatic inflammation mediated by IL-6. Although IL-6 is generally considered a protective cytokine. However, long-term exposure of the liver to infection leads to lowering IL-6 production had also been observed. In the kidney tissue of the artificially infected *L. Rohita*, the IL-6 production was corroborated with C3 production. But in muscle tissue IL-6 production shows sporadic changes shout for more detailed study of the related parameters.

Cytokines are group of signalling molecules that are responsible for the commencement and modulation of the inflammatory processes (Abo-Al-Ela, 2018). Interleukins (IL) and chemokines belongs to small secreted proteins superfamily and have the potentiality for the attraction of leucocytes at the injury site (Liu et al., 2007).

In bony and cartilaginous fishes, IL-1β was the first characterized interleukin (Zou et al., 1999; Bird et al., 2002). IL-1β is a proinflammatory cytokine that affects approximately all cell types in concert with various other proinflammatory cytokine like TNF (Huising et al., 2004). IL-1β plays a most important defensive role against invading microbes and also against injured tissue. It is having the ability for the development of immune responses by stimulating the lymphocytes and it can also enhance the production of different cytokine which will in turn on natural killer cells, lymphocytes and the macrophages. The primary source for the production of IL-1β are the macrophages. In fishes, the consecutive IL-1β expression was observed in various tissues like liver, kidney and spleen. However, the highest expression was observed in the spleen of the fishes (Tafalla et al., 2005). In the present study it had been found that the IL-1β expression was upregulated in the liver at the early stage of infection (12- 36 hpi). Kole et al., 2017 have found the similar result in the liver tissue of *L. rohita* infected with *E tarda*. In their finding it was shown that IL-1β expression was higher in the liver at the early stage of infection. However it had declined with the progression of time. Das et al., 2011 have also reported the early expression of IL-1β in *L. rohita* infected with *P sarana*. Zhou et al., 2018 have reported the same early expression of proinflamatory cytokines after infection of goldfish by gyrodactylids. However in the kidney tissue there was delay in IL-1β expression (72 hpi). Kole et al., 2017, have also reported the expression of IL-1β after 48 hpi and found maximum expression at 96 hpi. Whereas muscle cell did not participate in *inflammation* via IL-1β pathway. The early and delayed expression of IL-1β, explains their important function in defence against bacterial infection and recovery of host.