Chapter 4: Results..

4. Results

4.1 Microbiological isolation of *Klebseilla pneumoniae*

Under light microscope, gram staining of the isolated bacterial isolates revealed that all the isolates were pink colored rods (Fig. 12A). After 24hrs incubation of *Klebsiella* selective agar plates, the isolated bacterial strains were grown as mucoid purple colonies on the plates. Thus the result together indicating that the isolated bacterial strains are gram negative *K. pneumoniae* (Fig. 12B).

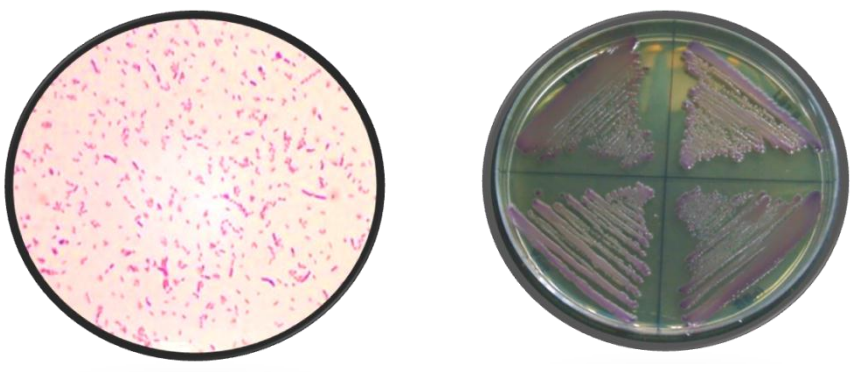


Fig. 12 Gram negative pink rods after gram staining (A) Purple and mucoid colonies of *K. pneumoniae* on selective agar (B).

4.2 Biochemical identification of the isolated strains

The biochemical test results of the isolated bacterial strains (K1- K10) for KB003- strip- 1 (Fig. 13A) showed positive results for lysine (isolate utilizes lysine for growth), urease (isolates had the capability to generate the urease enzyme that will help in hydrolization of urea), nitrate reduction (Isolate having capability of reducing nitrate), ONPG (Isolates were having β galactosidase enzyme), Voges- Proskauer's (Isolates producing acetonin), catalase (isolates having the ability to produce oxygen and water by cleaving H_2O_2), ornithine utilization (Isolates were having decarboxylase enzyme). However, the isolates showed negative results for phenylalanine deamination (isolates were unable to generate PPA and NH_3), H_2S

production (Isolates were not having the ability for reduction of compounds to sulphides), citrate (isolates unable to use citrate as carbon source), methyl red (inability to produce acids), indole (inability of the isolates for conversion of tryptophan).

The second strip (KB003- strip 2) (Fig 13B) for carbohydrate utilization showed positive result for all carbohydrate utilization tests *viz.* malonate (capability of the isolates for utilization of malonates as carbon source), esculin hydrolysis (it showed the ability of the isolates to hydrolyze esculin into esculetin & glucose) , arabinose (fermentation of arabinose by the isolates), adonitol (fermentation of adonitol as carbon source), rhamnose (fermentation of rhamnose as carbon source), cellobiose (fermentation of cellobiose as carbon source), xylose (fermentation of xylose as carbon source), saccharose (fermentation of saccharose as carbon source), raffinose (fermentation of raffinose as carbon source), trehalose (fermentation of trehalose as carbon source), glucose (fermentation of glucose as carbon source), lactose (fermentation of lactose as carbon source), melibiose (fermentation of melibiose as carbon source), sorbitol (fermentation of sorbitol as carbon source), mannitol (fermentation of mannitol as carbon source) (Fig. 14).

Fig. 13 *K. pneumoniae* biochemical test result KB003- strip1 (Fig. A) and carbohydrate utilization on KB003- strip2 (Fig. B)

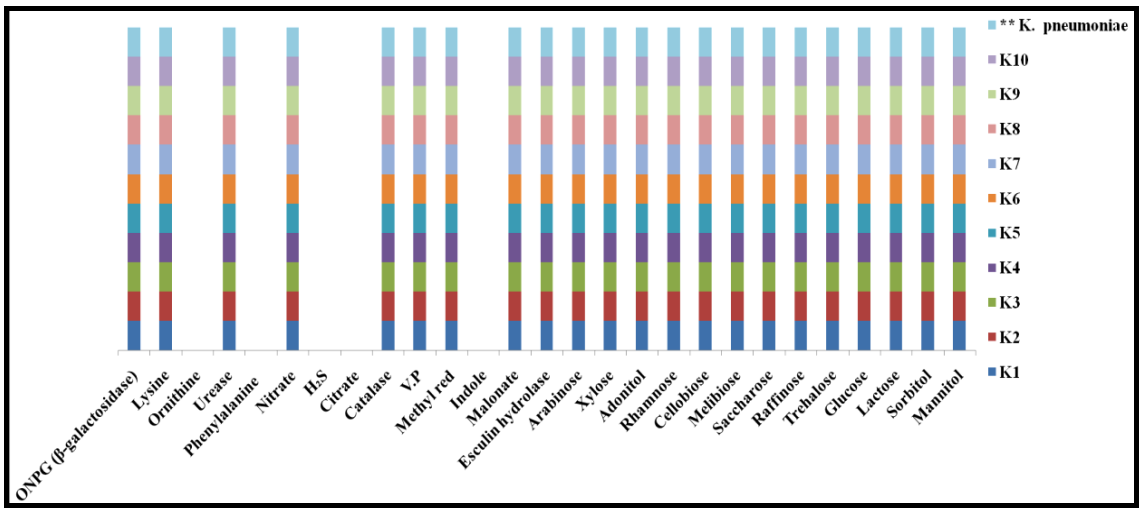


Fig. 14 Comparative biochemical test of isolated strains K1- K10 and ** *K. pneumoniae* (Alves et al., 2006)

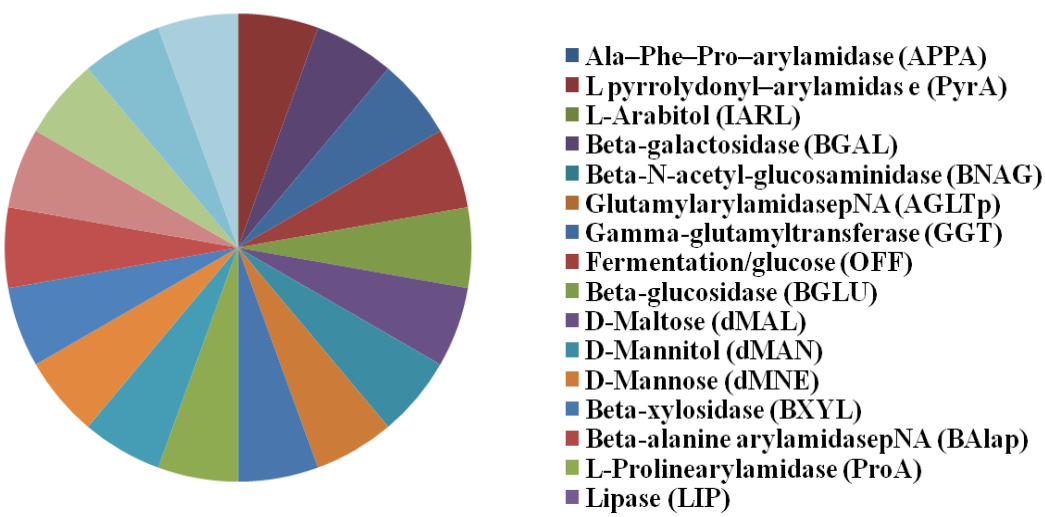


Fig. 15 Positive Biochemical test results of K1 in vitek compact 2.0

The biochemical test result of K1 in Vitek 2.0 revealed that it is positive for 18 biochemical tests form the above list pyrA, BGAL, GGT, OFF, BGLU, dMAL, dMAN, dMNE, BXYL, ProA, PLE, TyrA, dTAG, dTRE, AGLU, PHOS, LDC, GGAA (Fig. 15).

4.3 Genomic DNA and 16S rRNA PCR analysis

DNA isolated from the bacterial samples was loaded and visualized in 0.8% agarose gel. Intact DNA bands were obtained for all the isolates.

The 16S rRNA gene PCR amplified samples were loaded and visualized on 2% agarose gel. All the ten samples were amplified. Sharp and single bands of ˜1500 bp were observed on the gel (Fig. 16).

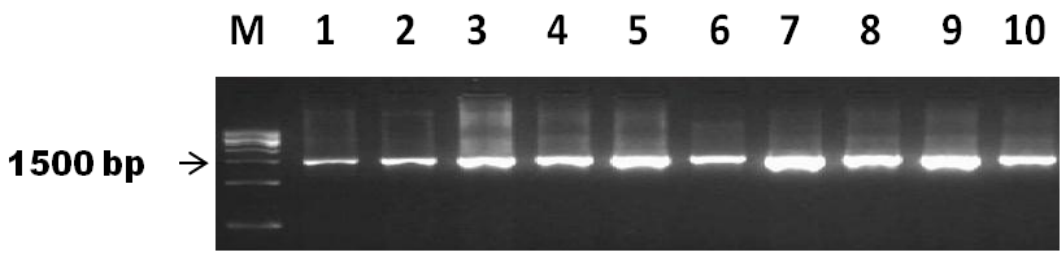


Fig. 16 Gel image of the PCR amplified samples. M= 100bp ladder, Lane 1- Lane 10= 16S rRNA gene of K1- K10 bacterial sample

4.4 Molecular identification and NCBI accession number

The amplified products were sequenced and contig was generated for each isolate. The contig for each isolate was blasted against NCBI Database. From the BLAST result it was found that the NCBI reported *K. pneumoniae* 16S rRNA gene sequence is having 100% identity with K1, K3, and K5. Whereas K2, K4, K6 isolates were having identity of 99% with globally reported *K. pneumoniae* and K7, K9 and K10 isolates were showing 97% identity with NCBI reported 16S rRNA gene of *K. pneumoniae.*

Table 6. NCBI accession number of the NCBI submitted *K. pneumoniae* strains

Sl. No.	*K. pneumoniae* strain ID	NCBI Genbank Accession No.	Host
1.	K1	KY003130	*L. rohita*
2.	K2	KU612260	*L. rohita*
3.	K3	KX010115	*L. rohita*
4.	K4	KX010116	*C. mrigala*
5.	K5	KX170832	*L. rohita*
6.	K6	MF680432	*L. rohita*
7.	K7	MF680483	*L. catla*
8.	K8	MF680516	*A. testudineus*
9.	K9	MF680540	*O. niloticus*
10.	K10	MF680539	*C. batrachus*

4.5 Phylogenetic analysis of the isolated bacterial strains

The first phylogenetic tree was constructed for the molecular and phylogenetic identification of K1 with different *Klebsiella* species. The constructed phylogenetic tree revealed that K1 is having a close evolutionary relationship with *Klebsiella pneumoniae* (NCBI accession number- KR856389). They were sharing the same node with highest bootstrap value of 100. Also K1 was sharing the same branch with *Klebsiella pneumoniae* KX350022 and KX350013. *Rickettsia conorii* (L36107) was found to be outgrouped from other *Klebsiella* species (Fig. 17).

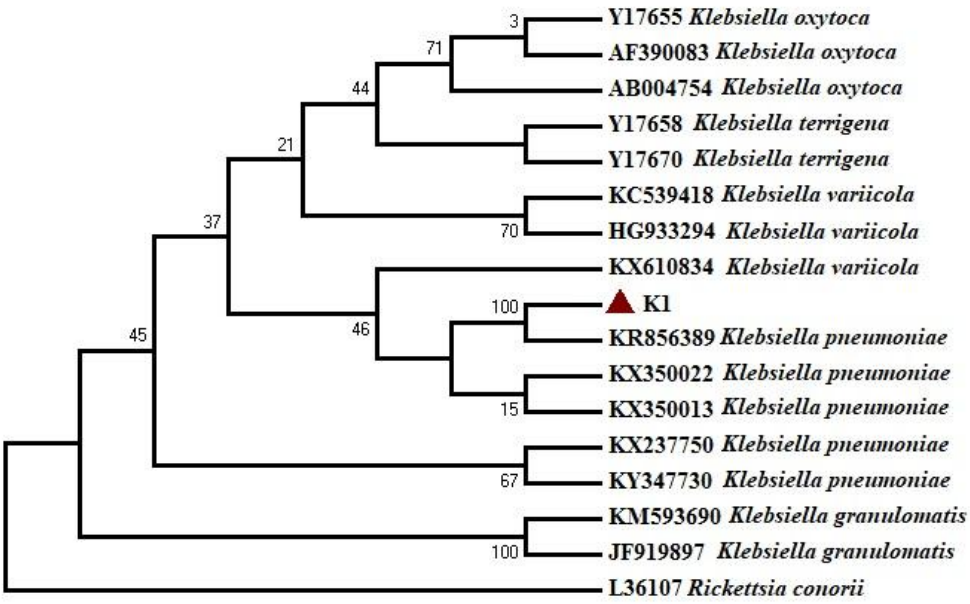


Fig. 17 Phylogenetic analysis of K1 isolate with other NCBI *Klebsiella* species based on 16S rRNA nucleotide sequences. K1 isolate have been designated with a shaded triangle.

In order to study the genetic diversity of the isolates, two more phylogenetic trees were constructed by using the maximum likely hood method. The aim of the first phylogenetic tree was to study the inter species diversity within the present isolates (K1- K10). Among the isolated strains from different geographical locations, the first phylogenetic tree (Fig. 18) showed that, the strains K5 isolated from North- 24 Parganas district and strain K10 isolated from Nadia district were sharing the same node and showing significant evolutionary relationship with highest bootstrap value. On the other hand, K3 isolate and K6 isolate from Burdwan district were found to be very close and also sharing the same node with a significant bootstrap value. However, K2 isolate from Burdwan district and K8 isolate from North 24 Parganas were also sharing the same node.

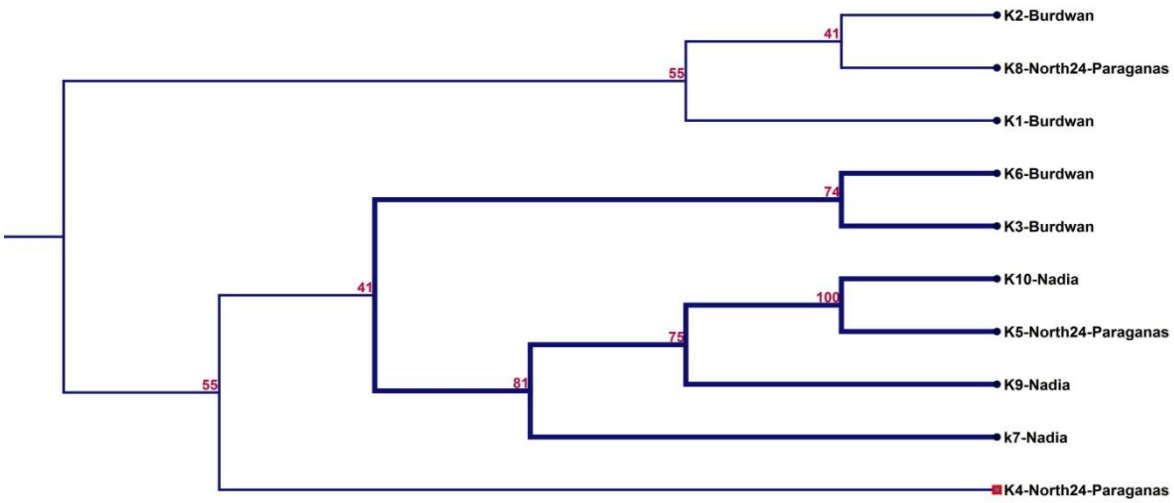


Fig. 18 Phylogenetic relationship between *Klebsiella pneumoniae* isolated from different aquaculture farmed diseased fishes of West Bengal

In order to understand the diversity of the present *K. pneumoniae* isolates with other globally reported *K. pneumoniae*, another phylogenetic cladogram was prepared using maximum likelihood method in CLC genomics workbench. 12 clusters were observed in the cladogram representing their evolutionary proximity (Fig. 19). In cluster I, K5 isolate from N- 24 Parganas and K10 isolate from Nadia were found to have highest similarity and were sharing the same node with a highest bootstrap value. They were also found to be evolutionary closer to the Indian *K. pneumoniae* reported strain (KM503154). Also the isolate K9 reported from Nadia was found to be evolutionary closer to the reported Sri Lankan strain (KY967368). In the IInd cluster, K1 strain reported from Burdwan was found to be evolutionary closer to Bangladesh strain (KX233848). In the IVth Cluster, K2 strain isolated from Burdwan was found to be phylogeneticlly closer to the Vietnam strain (KC213799) and K8 strain reported form N 24 Parganas was found to be sharing the same branch with USA strain (FJ823263), Vietnam strain (KC213799) and K2 strain. In the Vth cluster, K4 strain isolated from N 24 Parganas had evolutionary close relationship and was sharing the same node

with the Sri Lankan strain (K1985366).In cluster IX, K6 strain reported from Burdwan was evolutionary closer to the isolates reported from South Africa (KU946990), Bangladesh (KU992686), China (JX283459) and Indonesia (AB999796). K3 isolate reported from Burdwan and K7 isolate reported from Nadia had formed a separate cluster (cluster XI and cluster XII respectively) and they were found to be evolutionary diverse.

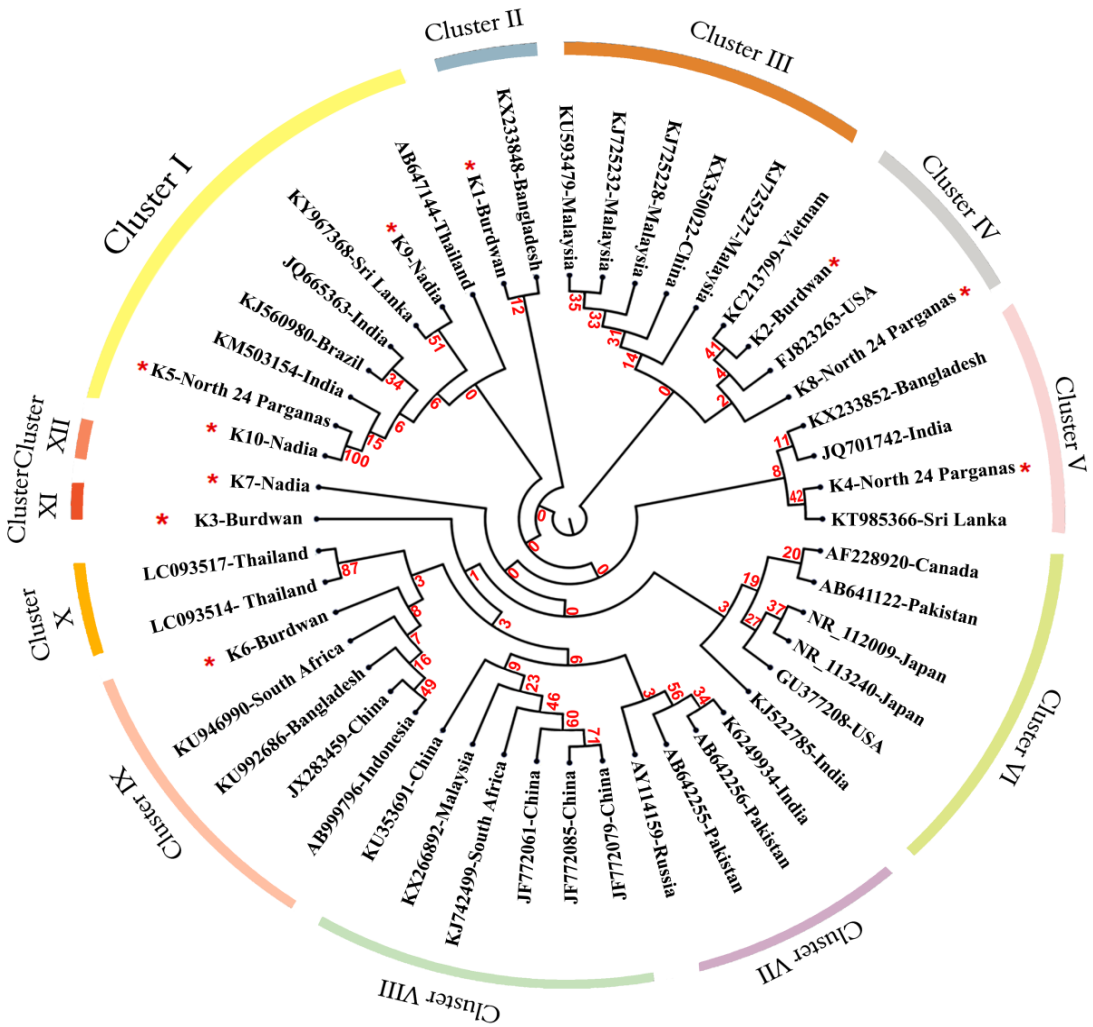


Fig. 19 Phylogenetic relationship of *Klebsiella pneumoniae* strains used in this study (*) with *Klebsiella pneumoniae* strains reported from different part of the world.

4.6 PCR ribotyping of *Klebsiella pneumoniae* isolates

Electrophoresis of the amplified products (16S- 23S ISR) showed that all the 10 isolates of *K. pneumoniae* have similar band patterns of around 550bp, 600bp, 700bp and 800bp size respectively (Fig. 20). In order to check the reproducibility of the technique, ribotyping of *Klebsiella oxytoca* ISR region using the same primer have produced band size of 600bp, 650bp, 750bp and 900bp. Whereas ribotyping of *Vibrio parahemolyticus* ISR region had produced band size of 650bp, 700bp, 800bp and 900bp respectively. PCR of the ISR region were carried out for three times and every time the gel eletrophoretic pattern was showing the same result. Thus the PCR results were constant and polymorphisms were not observed between the isolated strains.

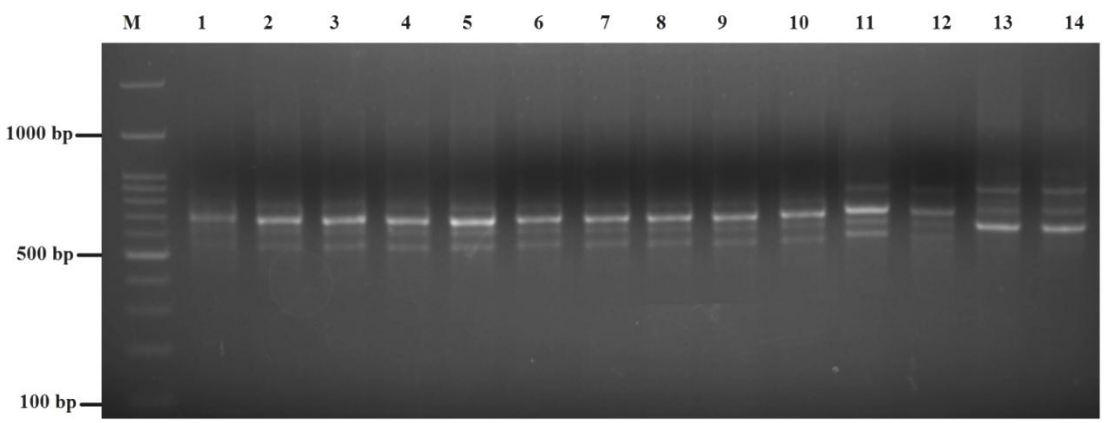


Fig. 20 PCR ribotyping gel image with M- 100 bp ladder, L1-L10- *K. pneumoniae* (K1- K10), L 11-12- *Klebsiella oxytoca*, L13- L14 *Vibrio parahaemolyticus*

4.7 Antibiogram sensitivity study and MAR index determination

The sensitivity test of the bacterial isolates against different antibiotics revealed that all the ten isolates were resistant for ampicilin, ceftazidime, dicloxacillin, rifampicin, imipenem, trimethoprim, cefipime, fosfomycin, amoxycilin and cefixime (Fig.21). However, in comparison to other isolates, K2 was resistant for both streptomycin and tetracycline. These are the most frequently used antibiotics in aquaculture farms. BothK5 and K10 isolate had shown resistance property against erythromycin. Resistant against doxycycline was shown by K10 isolate and K4 had shown resistant for chloramphenicol.

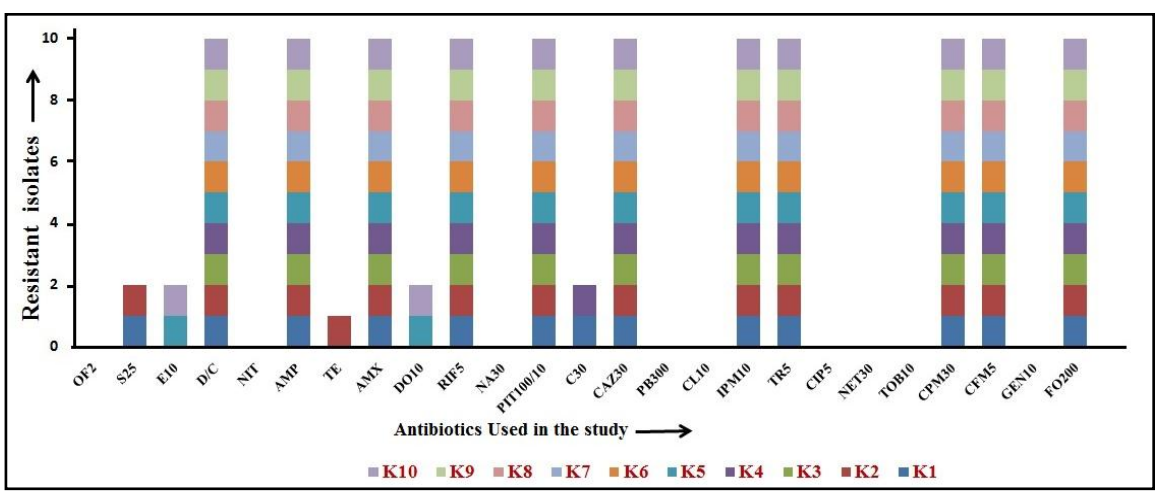


Fig. 21 Antibiotic study of K1- K10 strains against various antibiotics.

After determining the response of isolates towards different antibiotics, the calculated MAR value was found to be 0.468.

Total number of antibiotic resistance case= 119, **Total number of antibiotics**=25,

Total number of isolates= 10 **MAR index**= 119/ 25*10= 0.476

4.8 Solid hemolysin assay and Liquid hemolysin assay

Bacterial hemolysis of RBCs shows the pathogenicity of the bacterial species. After incubating the isolates for 48 hrs on blood agar plates, a clear and colourless zone of hemolysin were observed on the plates. The plates were showing colorless and clear zone, reflects complete RBCs lysis (Fig. 22A). Typical β- hemolysin pattern were observed for each isolates.

In early logarithmic phase, the isolate was showing the highest haemolytic activity (10 – 12 h). However, with the increasing time period the haemolytic activity of the isolate was decreasing and after 36 hpi, the hemolytic activity of the isolate became stationary. The maximum hemolytic activity was during (Fig. 22B).

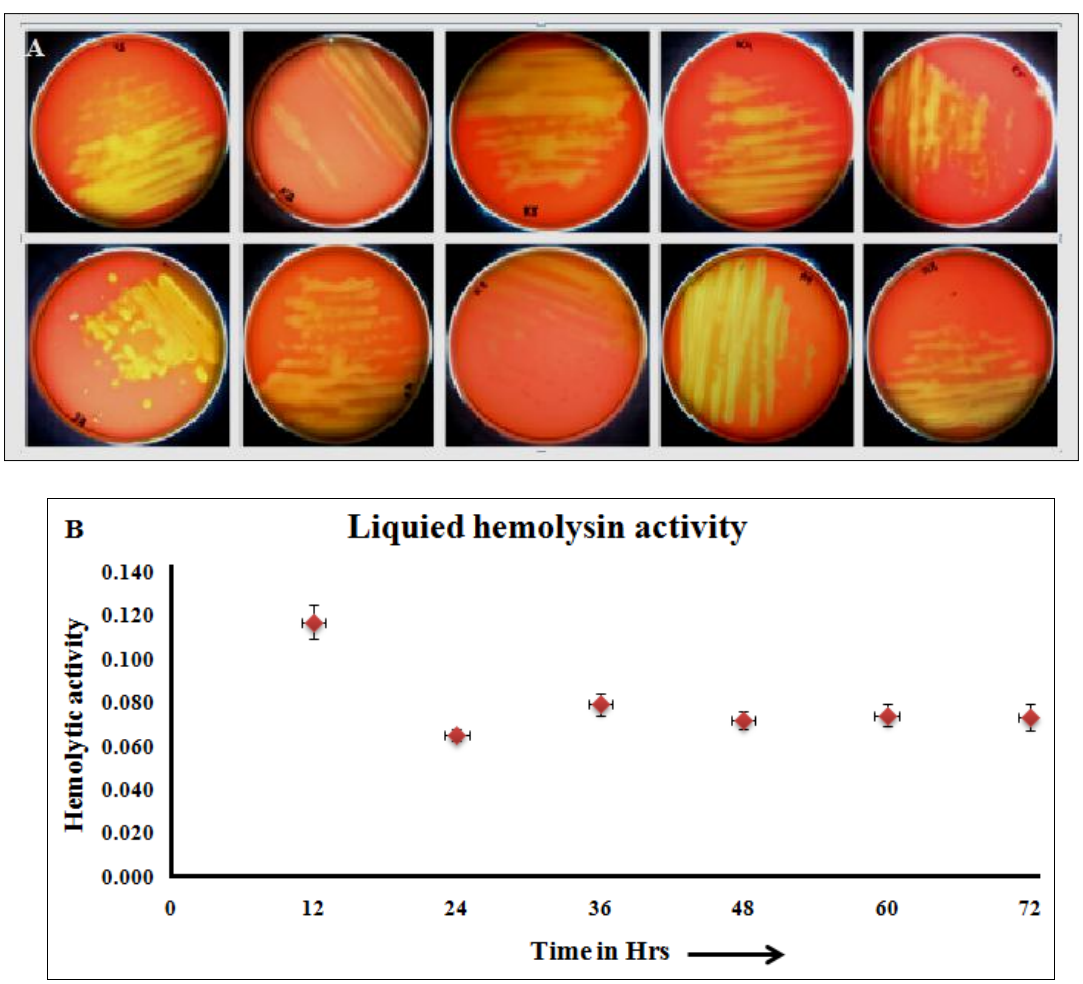


Fig. 22 Solid hemolysin assay on bloog agar plate supplemented with sheep blood (A); (B) Liquid hemolysin assay on sheep blood R.B.C.

4.9 *In-vivo* pathogenicity test

After injecting *L. rohita* with different concentration of K1 isolate, the cumulative mortality rate was shown in Fig. 23. After 10 days post injection of the control with NS, mortality was not being observed. However, the challenged fishes have developed haemorrhages and reddening at the injected sites (Fig. 24). Experimental animals injected with higher concentration of bacteria had died at the early infection stage. Bacterial pathogen was re-isolated from blood, liver and kidney of the infected animals and was found to be similar as the injected strain.

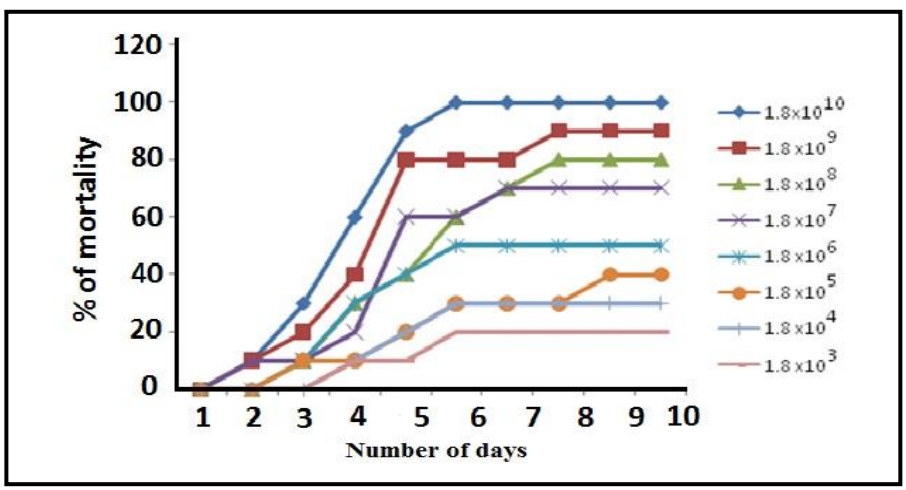


Fig. 23 Mortality rate of *Labeo rohita* after artificially challenged with K1 at different concentrations

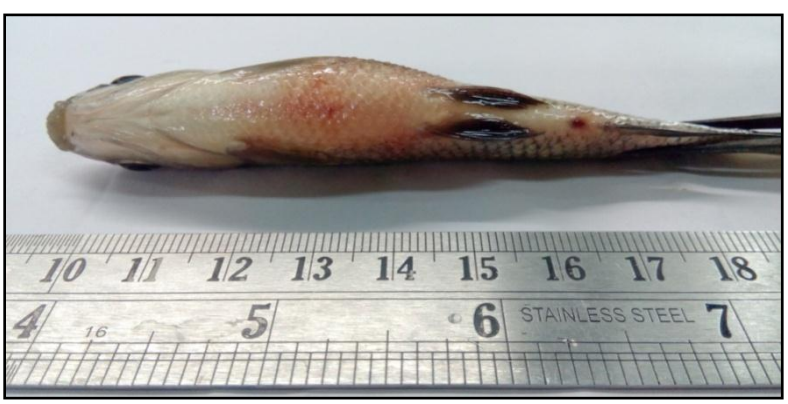


Fig. 24 Haemorrhages and reddening developed by fishes after the interperitonial region

4.10 LD_{50} determination of *Klebsiella pneumoniae*

The dose of *K. pneumoniae* required for 50 % mortality of the *L. rohita* was calculated by the data provided in table 7.

Table 7. LD_{50} estimation of *K. pneumoniae* in *L. rohita*

Bacterial concentration	No. of animals Died	No. of animals survived	Cumulative total				
			Died	Survived	Total	Ratio (Died/ Survived)	Mortality percentage (%)
$1.8X\ 10^{10}$	10	0	48	0	48	1	100
$1.8X\ 10^{9}$	9	1	39	1	40	0.975	97.5
$1.8X\ 10^{8}$	8	2	31	3	34	0.91176	91
$1.8X\ 10^{7}$	7	3	24	5	29	0.82759	82.7
$1.8X\ 10^{6}$	5	5	19	8	27	0.7037	70
$1.8X\ 10^{5}$	4	6	15	11	26	0.57692	57
$1.8X\ 10^{4}$	3	7	12	13	25	0.48	48
$1.8\ X\ 10^{3}$	2	8	10	15	25	0.4	40

$$\text{Proportionate distance} = \frac{\text{\% of mortality above 50\%- 50}}{\text{\% mortality above 50\%- \% mortality below 50\%}}$$

By using the Reed and Muench method, LD_{50} for *K. pneumoniae* was found to be $1.05\ X\ 10^{6}$ CFU /fish.

4.11 Histopathological findings

In the kidney of the artificially infected fishes, ultrastructural alterations in the renal tubules and glomeruli were observed (Fig. 25A). Also vacuolation were noticed in the kidney tissue (Fig. 25B).

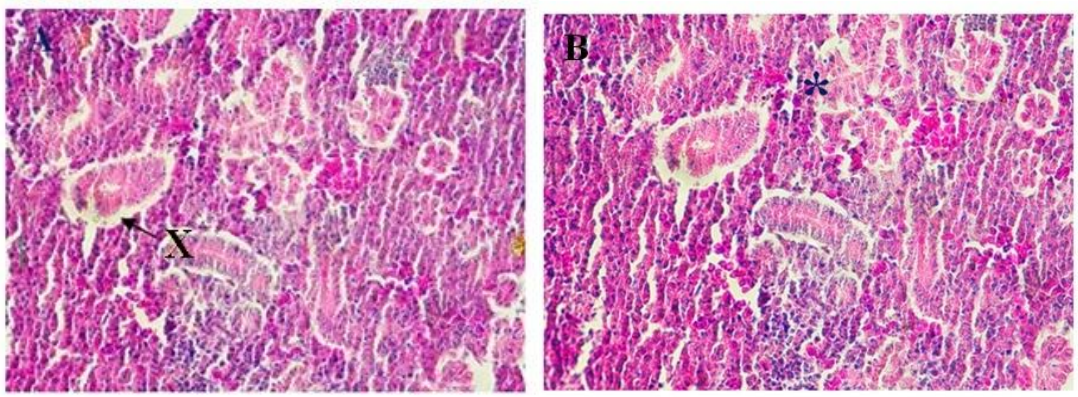


Fig. 25 A–B. Photomicrograph of artificially infected *L. rohita* Kidney [A]: tissue necrosis, ultra structural alterations in glomeruli and renal tubules (*); [B]: vacuolation in kidney (X) (H & E staining; 60X)

Ultrastructural changes like necrosis, vacuolation and hepatocytes disruption were observed in the liver tissue (Fig. 26A). Also melanomacrophages center were evident in the infected liver tissue (Fig. 26B).

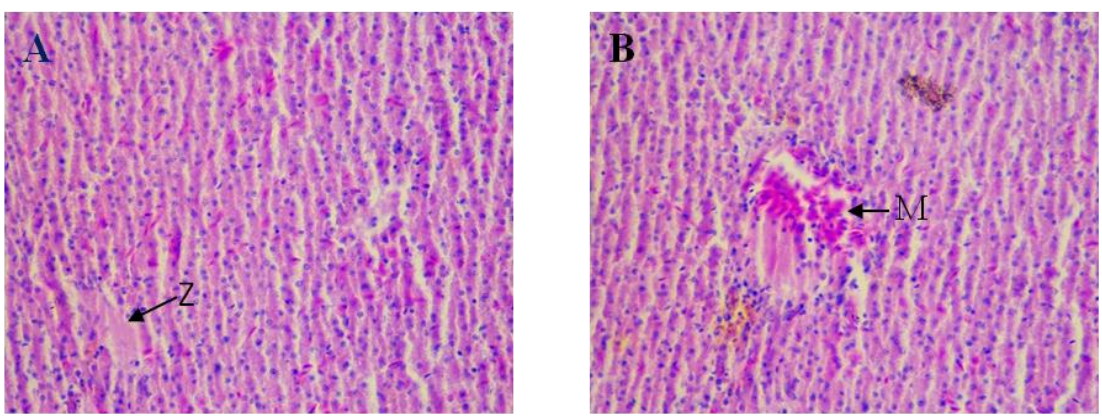


Fig. 26 A–B. Photomicrograph of artificially infected *L. rohita* liver showing [A]: hepatocytes disruption, vacuolation and necrosis (Z); [B]: melanomacrophages center (M) were observed (H & E staining; 60X)

4.12 String Test for Hypermucoviscosity

After overnight incubation, on stretching the colony with a sterile loop a viscous string of > 5 mm was formed (Fig. 27). Thus showing the bacterial isolates were hypermucoviscous (HV) in nature.

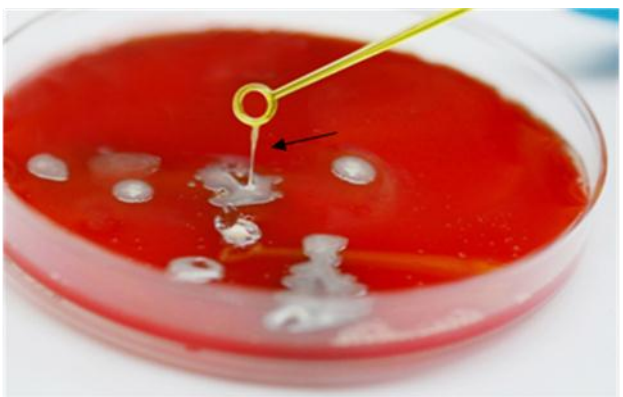


Fig. 27 Formation of viscous string by *Klebsiella pneumoniae* on blood agar plate.

4.13 Molecular identification of the virulent genes

Out of the 12 virulent genes, eight virulent genes (*fim*A, *fim*H, *mrk*A, *ure*, *uge*, *ent*B, *wab*G, *ecp*RAB) had got amplified (Fig. 28). The full length gene sequence of obtained was then submitted to the NCBI database (Accession Number: MK241970, MK303548, MK264337, MK241549, MK264338, MK264340 MK264339).

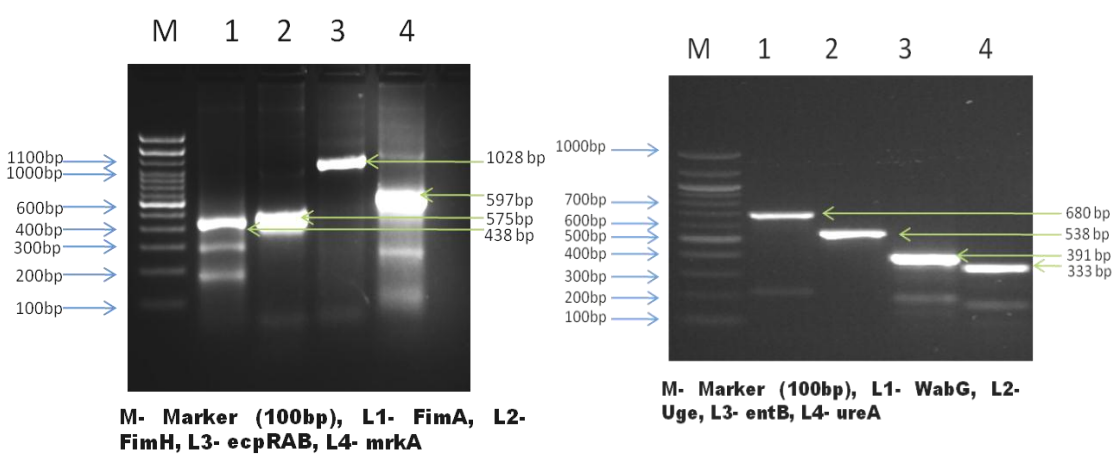


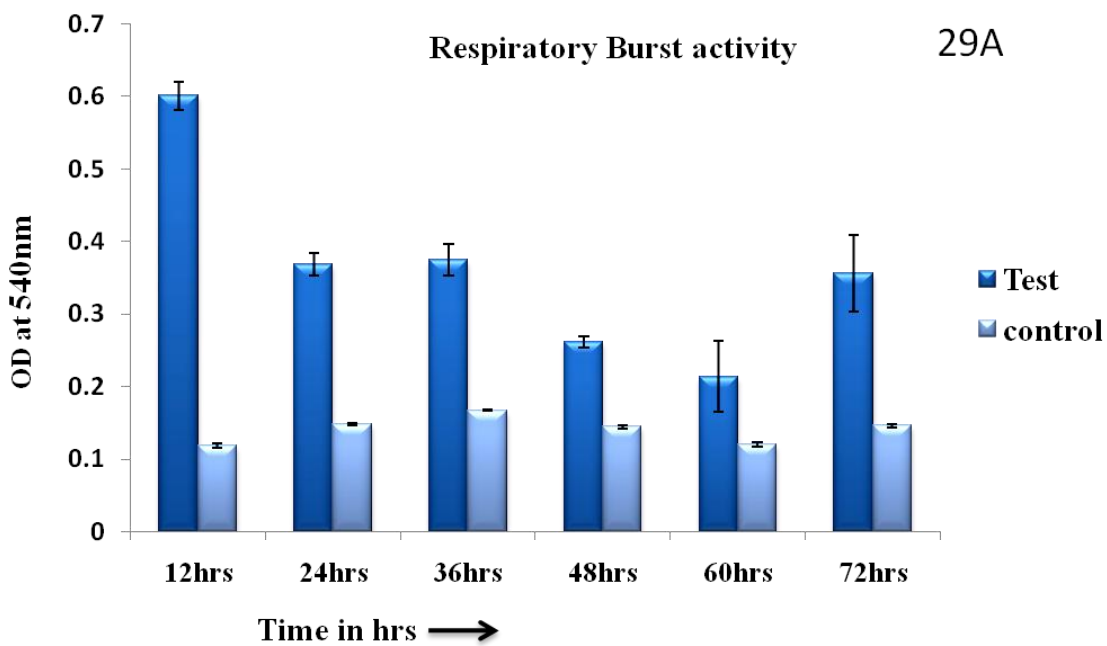
Fig. 28 Agarose gel (2%) image of the amplified virulent genes

4.14 Innate immunity parameters of Non specific immune response

Infected group and control group were sampled at different hours.

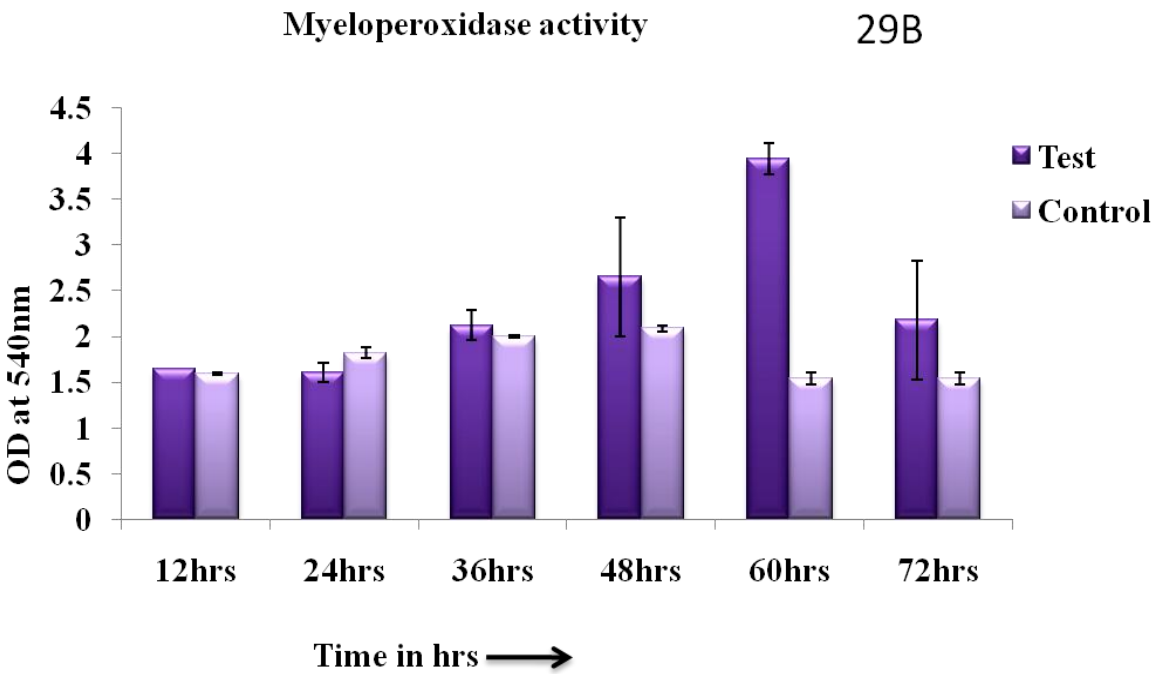
4.14. 1 Respiratory burst assay

Throughout the process of infection, the superoxide production was significantly ($p < 0.05$) higher in infected group fishes on comparison with the control group. Maximum production of reactive oxygen was observed at the early infection stage (12 hpi). However in the control fishes, no significant variations in superoxide production have been observed (Fig. 29A)



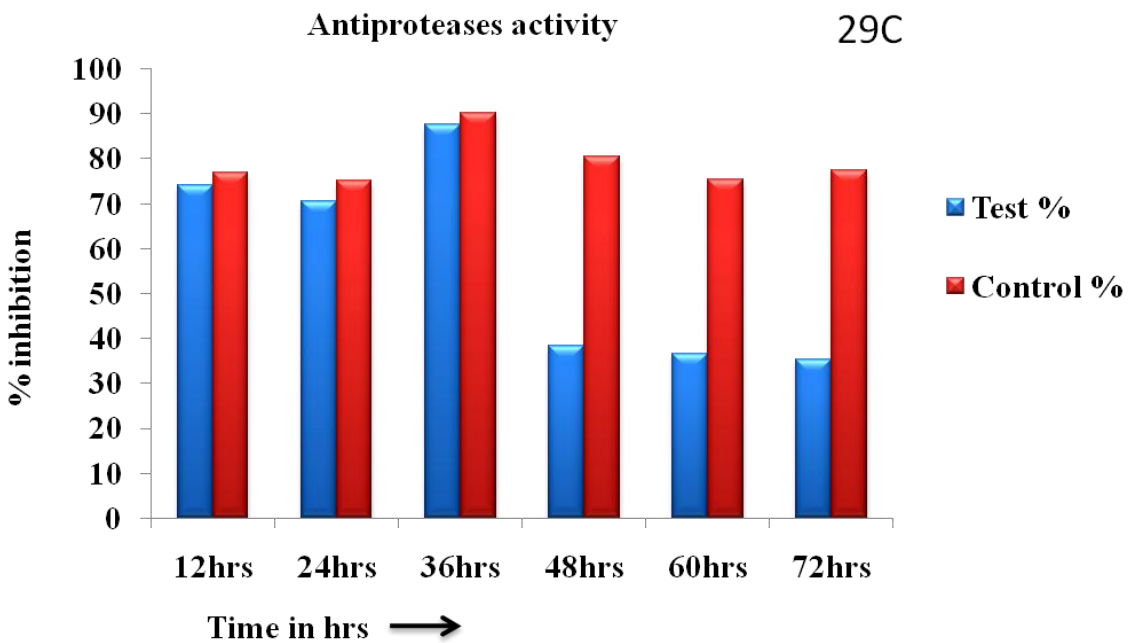
4.14. 2 Myeloperoxidase (MPO) activity

During the experimental process after 36 hpi, higher MPO activity was evident in the infected groups (Fig. 29B). In infected group, maximum MPO activity was noticed at 60 hpi. However no significant variation was observed in the control group.



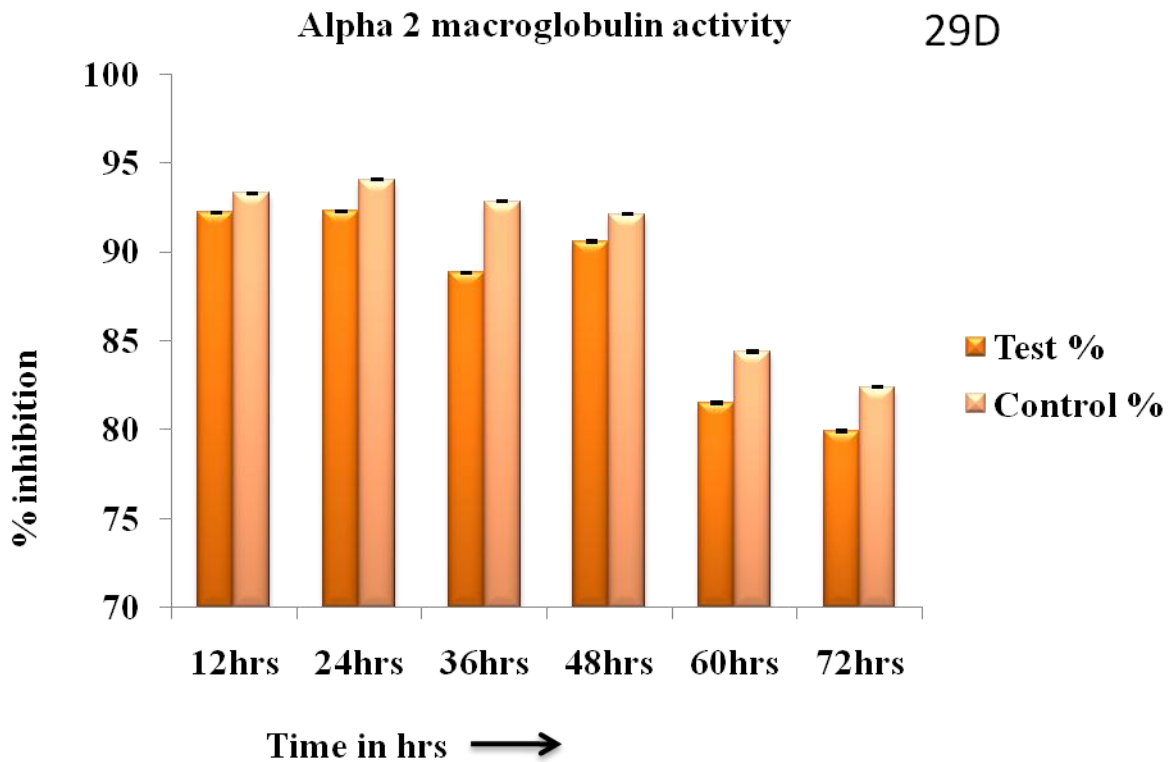
4.14. 3 Total antiproteases activity

On comparison with the control group, a gradual reduction of antiprotease activity was observed in the infected samples. Till 36 hpi, no significant differences were observed in both the control and infected groups. However, after 36 hours of infection, a significant ($p < 0.05$) higher total antiprotease activity was evident in the control group on comparison with the infected group (Fig. 29C).



4.14. 4 α-2 Macroglobulin assay

Throughout infection process, a lower α-2 M activity was evident in the infected group on comparison with control group. At advanced infection stage, after 36 hpi a significant ($p < 0.05$) lower α-2 M activity of was seen in the infected group (Fig. 29D).



4.14. 5 Lysozyme activity

Upto 12 hpi, serum lysozyme activity of control group was found to be higher than the infected group. However, with the progression of infection the lysozyme activity of infected group was found to be increasing. Highest lysozyme activity was observed at 48 hpi. A significant ($p < 0.05$) higher lysozyme activity of the infected group was evident after12 hpi (Fig. 29E).

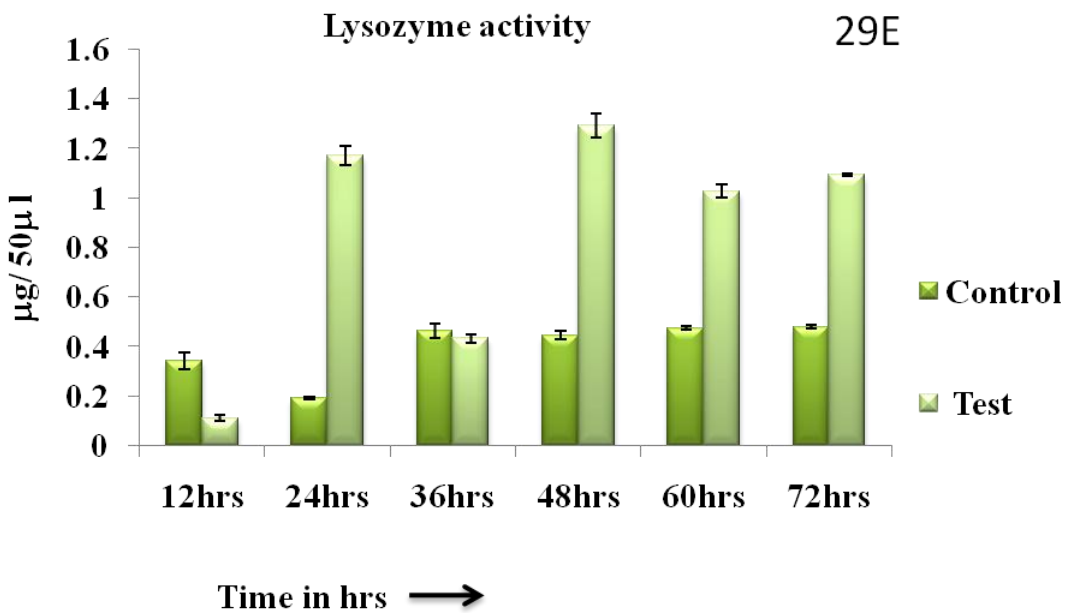


Fig. 29 Non-specific immune system response of *Labeo rohita* after *Klebsiella pneumoniae* infection at different hours; a. Respiratory burst activity, b. Myeloperoxidase activity, c. Antiprotease activity, d. Alpha 2-macroglobulin, e. Lysozyme activity. Data are presented as mean ± S.E.

4.15 Specific immune response

4.15. 1 C3 gene expression level

Significant upregulation (P< 0.05) of C3 gene have been observed in the liver after 12, 24 and 60 hpi. However, at 72 hpi the C3 gene expression level had reached basal level. In case of Kidney, at the early stage of infections (12 hpi and 24 hpi), the C3 level was down regulated but after 36 hpi there was significant upregulation (P< 0.05) of C3 gene. In case of Muscle, C3 gene had upregulated upto 60 hpi. The maximum expression was observed at 24 hpi. However, no significant variation (p> 0.05) was noticed in the C3 gene expression.

4.15. 2 IL6 gene expression level

In the liver, the IL6 expression level was upregulated upto 36 hpi. But at 48 hpi infection the expression level had reached its basal level and then downregulated. However, in the liver significant variation (p> 0.05) was not evident in IL6 expression. In kidney it had been observed that IL6 level was downregulated at the early stages of infections (12- 36 hpi). However significant upregulation (p< 0.05) have been observed from 48hpi upto 72 hpi. In case of muscle tissue, IL6 level was downregulated during early phases of infection, however significant upregulation (p< 0. 05) was observed at 48 and 72 hpi.

4.15. 3 IL- 1β gene expression level

The IL-1β level of liver was significantly upregulated (p < 0.05) at the early stages of infection however at the post infection level it had reached its basal level at 72 hpi. In Kidney tissue, upto 48 hpi there was significant downregulation of the gene however significant upregulation (p < 0.05) was observed at 60 and 72 hpi. In case of muscle,

the IL1β was found to be downregulated throughout the experiment. No significant variation was observed ($p < 0.05$).

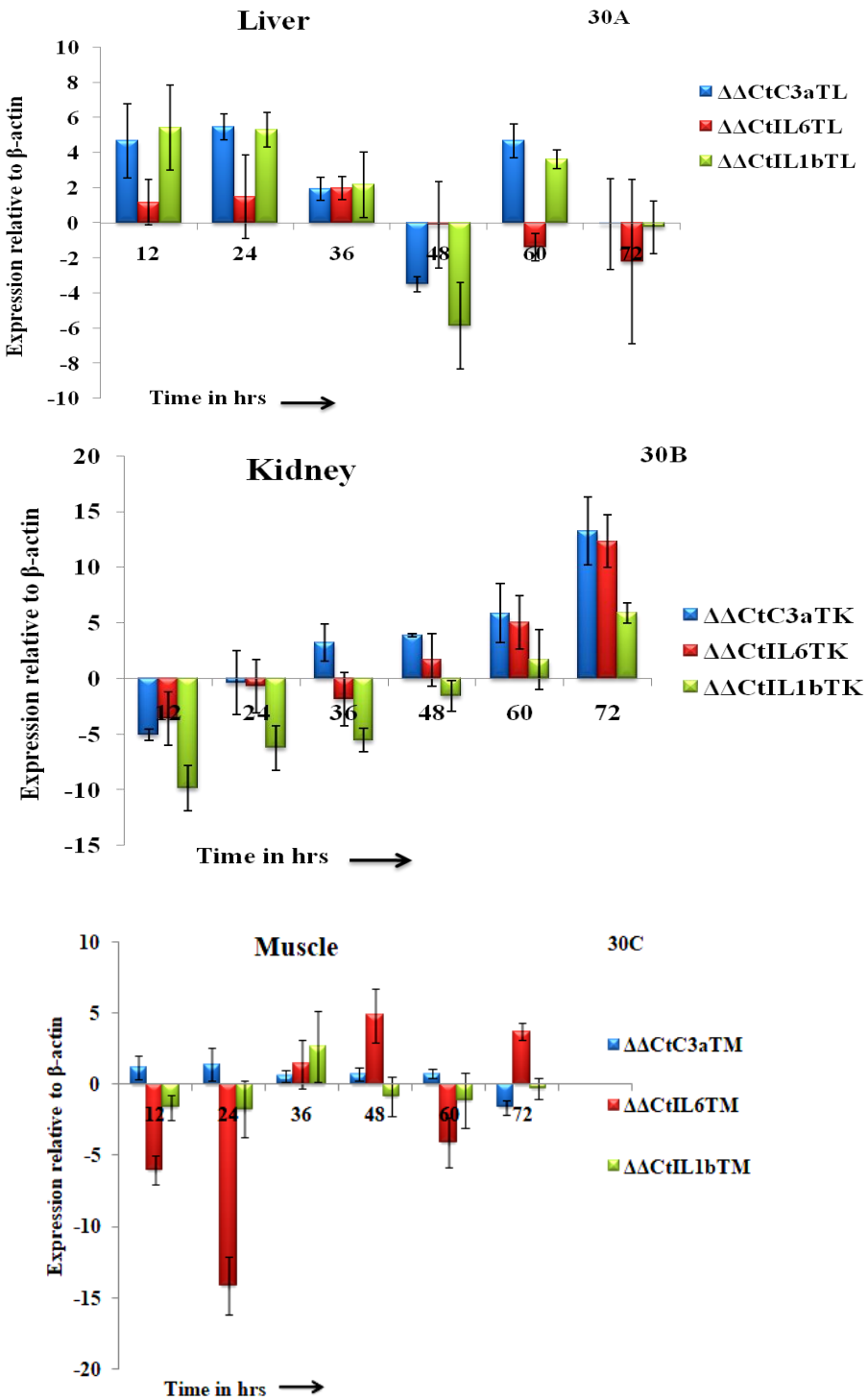


Fig. 30 (A-C) Relative Expression of C3, IL6, IL-1β genes relative to β– actin gene in (A) Liver, (B) kidney and (C) Muscle of *L. rohita* infected with *K. pneumoniae*. Error bars indicates mean values (±S.E.) of three samples.