Chapter 3: Materials and methods..

3. Materials and Methods

3.1. Materials

3.1. 1. Equipments

Autoclave (Amlagamated pvt. limited), weighing balance (Essaeteraoka limited), shaking incubator, hot air oven (Labtech), biosafety cabinet (Haier biomedical, India), forceps, scalpel, scissors, glass slide, embedding cassette and embedding molds (Hi media), micropipette (Eppendorf, USA), cold centrifuge (HERMLE, USA, Model no-Z323K), thermal cycler (Applied Biosystems, USA, Model no-GeneAmp 9700), gel electrophoresis apparatus (Bio-Rad, USA, Model no-wide mini-sub cell GT system), power pac (Bio-Rad, USA), gel documentation unit (Bio-Rad, USA, Model no-Geldoc XR + system), milli Q water unit (Merck Millipore, USA, Model no-Milli Q Advantage A10), spectrophotometer (Thermo scientific, USA, Model no-Genesys 10S UV-Vis spectrophotometer), pH meter (Fisher scientific, USA, Model no-Accumet AB15E), vortex (Tarson, India, Model no.- Spinix 3020), micro centrifuge (Tarson, India, Model no.- Spinwin 1020), magnetic stirrer (Tarson, India, Model no.- 6020), freezer (4°C, -20°C, -80°C) (Haier biomedical, India), microscope (Nikon), microtome (Leica EG 1140H, Germany), Ice flaking machine (SR lab instruments), and Real time PCR (Roche Light Cycler 480).

3.1. 2. Media	
• Hi Crome Klebsiella Selective HiVeg Agar Base	• Blood Agar Base (Infusion Agar)
• Tryptone Soya Broth (TSB)	• MacConkey Agar
• Tryptone Soya Agar (TSA)	• Brain Heart infusion broth (BHI)

3.1. 3. Chemicals

Genomic DNA isolation	mRNA isolation	Qualitative PCR	Quantitative RT-PCR	Histology	Agarose gel electrophoresis	Non specific immune study
Tris-EDTA buffer Proteinase K Sarkosyl Phenol Chloroform Isoamyl alcohol sodium acetate Ethanol	• Diethyl pyrocarbonate (DEPC) • RNA zap • RNA later • Trizol • Chloroform • Isopropanol • Ethanol • Nuclease free water.	• Nuclease free water • dNTPS • PCR buffer • Forward and reverse primers • Taq DNA polymerase • $MgCl_2$.	Sybr green master mix Forward and reverse primer Nuclease free water	• 10% neutral buffer formaldehyde (NBF) • Egg albumin • Ethanol (different graded) • Distilled water • Harris' Hematoxylin • Eosin • Xylene	• Agarose, • Tris actetate EDTA buffer (TAE buffer) • Ladder (100 and 500 bp) • Loading dye • Ethidium bromide (EtBr)	• Nitro-blue tetrazolium (NBT) • Dimethyl formaamide • Hanks balanced salt solution • 3,3,5,5 tetramthylbenzidinehydrochloride (TMB), • Hydrogen peroxide (H_2O_2) • H_2SO_4 • Trypsin, • Tris-HCl, • Na- benzoyl-DL-Arginine p-nitroanilide (BAPNA) • Acetcic acid • Phosphate buffered saline (PBS) • Lysozyme • Sodium acetate • Micrococcus lysodeilticus

3.2 Methodology

3.2.1 Sampling of diseased fish

During the year 2014-16, random samplings were carried out quarterly in fresh water aquaculture farms of Nadia, North 24 parganas, east and west Burdwan district's of West Bengal for isolation of disease causing pathogenic bacteria. Five (5) farms from each district were selected for sampling and 5-6 fish samples were collected from each farm. A total number of 610 fishes were collected for the present study. Diseased moribund fish Samples of *Labeo rohita, Labeo catla*, *Cirrhinus mrigala*, *Anabas testudineus*, *Clarias batrachus* and *Oreochromis niloticus* of various sizes were exhibiting red spots, hemorrhages and lesions near the operculum, dorsal fin, intraperitoneal region and also at anal position (Fig. 5A-5D).

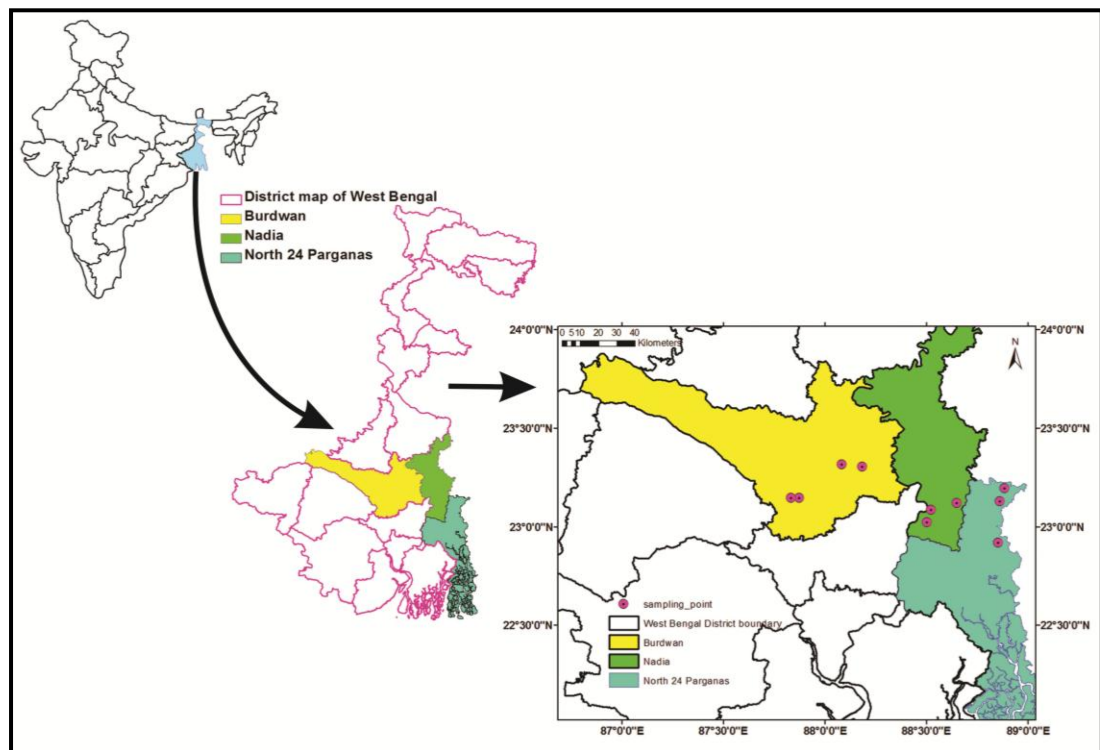

Fig. 4 Geographical location of sampling from aquaculture farms of West Bengal, India

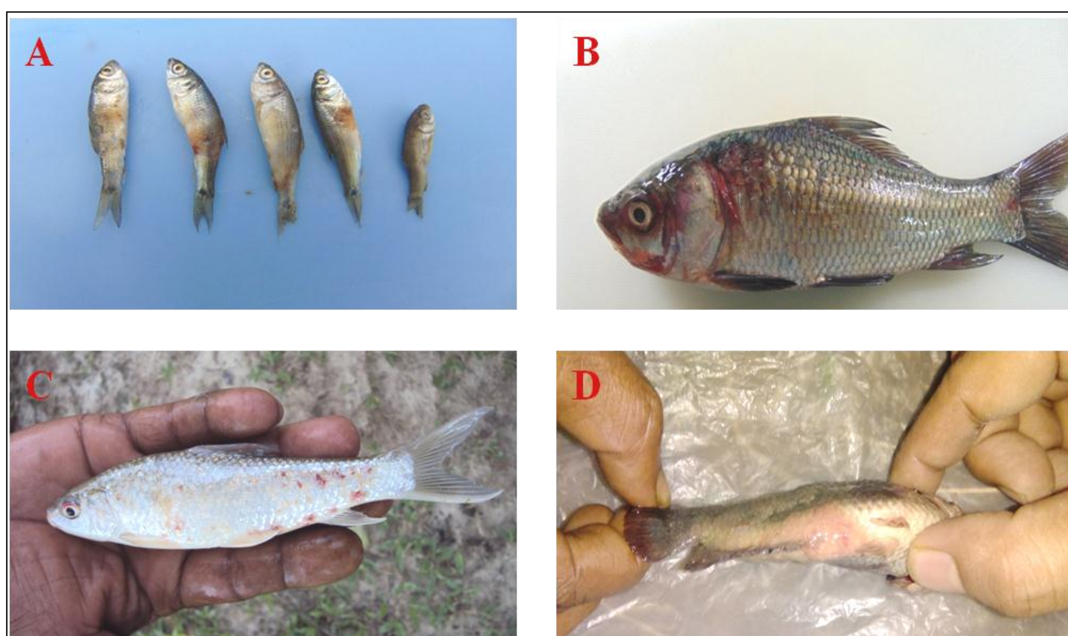

Fig. 5 Haemorrhages were observed in *L. rohita* fingerlings (A); haemorrhages were observed near the operculum of *L. catla* (B); red spots were observed in the anal and interperitonial region of *C. mrigala* (C); haemorrhages were observed at the interperitonial region of *A. testudineus* (D)

3.2.2 Preparation of different Medias

TSA mediaHi-media M290	
Components	**Amount**
Tryptone Soya Agar	4 gm
Milli-Q water	100 ml

The media was mixed, autoclaved and distributed in fresh sterilized Petri plates.

TSB mediaHi-media M011	
Components	**Amount**
Tryptone Soya Broth	3 gm.
Milli-Q water	100 ml.

3.00 gm of the above media was added to 100 ml of milli Q water. The media have been dissolved and distributed in test tubes. The tubes were further autoclaved.

Brain Heart infusion - Hi-media M1582	
Components	**Amount**
MacConkey Agar	3.70gm
Milli-Q water	100 ml

After dissolving the media, it was distributed into test tubes. The tubes were autoclaved.

MacConkey Agar- Hi-media M1582	
Components	**Amount**
MacConkey Agar	4.65 gm
Milli-Q water	100 ml.

After mixing the media, it was autoclaved and poured into sterile petriplates.

Hi-ChromeKlebsiella Selective Agar Base-Hi-media MV1573	
Components	**Amount**
Klebsiella Selective Agar	4.08 gm
Milli-Q water	100 ml.

In 100 ml of milli Q water, 4.08 gm of the above media was added. The media have been boiled until it gets dissolved and 400 µl of Klebsiella selective supplement (FD 225- Hi media) have been added.

Blood Agar Base (Infusion Agar)- Hi-media M073	
Components	**Amount**
Blood Agar Base	4.00 gm
Milli-Q water	100 ml.

After mixing the media, it was autoclaved and allowed to cool (45- 50°C). After cooling 5 ml of defibrinated sterile sheep blood was added and mixed. The media was poured in petriplates.

3.2.2.1 Preparation of reagents

A. 1 M Tris-HCl

Sl. No.	Components	Amount
1.	Tris-HCl	12.114 gm
2.	TDW (dH_2O)	100ml
3	pH	7.5

B. 0.5 M EDTA

Sl. No.	Components	Amount
1.	$Na_2EDTA.2H_2O$	18.6 gm
2.	TDW (dH_2O)	100ml
3	pH	8

C. 10:1 TE buffer

Sl. No.	Components	Amount
1.	1 molar Tris-Cl (pH 8.0)	1
2.	0.5 molar EDTA	0. 200
3.	TDW (dH_2O)	98.8
4	pH	7.5
	TOTAL	**100**

D. **10 % Sarkosyl**

10 gm of sarkosyl was added to 100ml of TDW and stored at 4°C

E. **Proteinase K (20mg/ ml)**

In 10 ml of TDW, 200mg of proteinase K was dissolved properly. Final stock was maintained at -10° C.

F. **0.3M CH_3COONa**

In 100 ml of TDW, 2.46 gm of sodium acetate was dissolved and maintained at 4°C

G. **70 % ethanol**

30 ml of TDW was dissolved in 70 ml absolute ethanol to prepare 70 % ethanol. It was stored at 4°C freezer.

H. Ethidium Bromide solution

10 mg EtBr was dissolved in 1.0 ml TDW and then continuous shaking for 24 hrs.

I. Preparation of TAE (50 X)buffer

Sl. No.	Components	Amount
1.	Tris base	242 gm
2.	Glacial acetic acid	57.1 ml
3.	0.5 M EDTA (pH 8.0)	100 ml
4.	TDW (dH_2O)	Upto 1000 ml
	Total	**1000 ml**

J. Preparation of TAE (1 X)buffer

Sl. No.	Components	Amount
1.	50X TAE	20 ml
2.	TDW (dH_2O)	980 ml
	Total	**1000 ml**

K. 0.8 % Agarose gel

0.8 gm of agarose was heated in 100 ml 1 X TAE buffer until a clear and transparent solution was formed. Once the mixture was cooled down to 45-50°C, 2-3 µl of EtBr was mixed in that solution and poured it into the gel casting tray to solidify the gel.

L. 1.8 % Agarose gel

1.8 gm of agarose was heated in 100 ml 1 X TAE buffer until a clear and transparent solution was formed. Once the mixture was cooled down to 45-50°C, 2-3 µl of EtBr was mixed in that solution and poured it into the gel casting tray to solidify the gel.

M. 30 % ethanol

30 ml of absolute ethanol was dissolved in 70 ml of TDW to prepare 30 % ethanol.

N. **50 % ethanol**

50 ml of absolute ethanol was dissolved in50 ml of TDW in to prepare 50 % ethanol.

O. **90 % ethanol**

90 ml of absolute ethanol was dissolved in 10 ml of TDW in to prepare 50 % ethanol.

P. **10% NBF (Neutral buffered formalin)**

Chemical	**Quantity(for 500ml)**
37% Formaldehyde	50 ml
Distilled Water	450 ml
Na_2HPO_4	3.25 gm
NaH_2PO_4	2 gm

Q. **Acid alcohol**

1 ml HCl/H_2SO_4 in 100 ml of absolute alcohol

R. Scotts tap water

1.4 gm of $NaHCO_3$ and 8 gm of $MgSO_4$ in 400 ml in water.

3.2.3 Isolation of causative pathogen from diseased sample

- Moribund diseased fishes showing clinical signs of hemorrhages and red spots on their body were anesthetized by using MS 222 (Hi media) (150 ppm).
- For each infected fish sample, blood was collected from the tail portion by using heparinized syringe.
- 2-3 drops of blood were inoculated in TSB media tubes and cultivated in shaking incubator for 24 hrs at 37°C.
- 9 sterilized test tubes were marked from 10^{-1}upto10^{-9}
- In each test tube, 9 ml of autoclaved water added.
- Tubes were autoclaved for sterilization.
- Then 1 ml of culture was poured into the 10^{-1} labeled test tube and mixed well and then serially diluted upto $10^{-9.}$
- Sterilized glass spreader was used to spread 100µl of culture from $10^{-7}$to 10^{-9} diluted tubes on TSA plate.
- Further, overnight incubation of the culture spreaded plates were done at temperature of 37 ºC.
- After formation of single colonies on TSA plates, each colony was transferred to MacConkey agar and *Klebsiella* selective agar plate.
- The pure culture grown on *K. pneumoniae* were sub cultured in Tryptic Soya Broth (TSB) tubes
- Overnight grown pure cultures were designated with codes *viz.*K1- K10 and were maintained in 20% glycerol stock at a temperature at -20 ºC.

3.2.4 Biochemical identification of bacterial isolates

Using Hi- Media KB-003 biochemical strips, the bacterial strains were primarily identified. For K1 strain, Additional in 38 biochemical tests were carried out in VITEK 2 (Bio- Merieux, France) compact automated bacterial identification system.

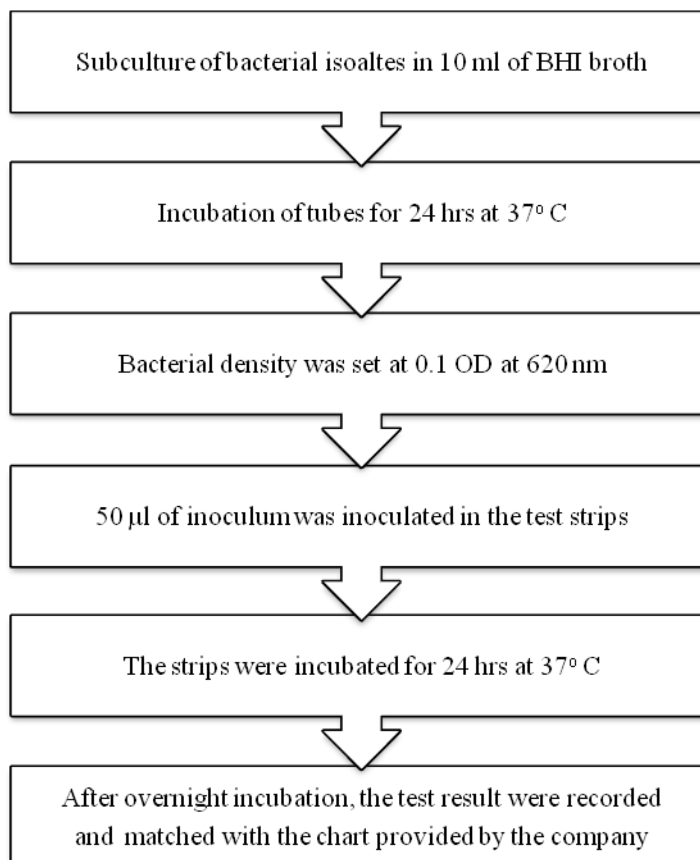

Fig. 6 Schematic flow chart of biochemical tests

3.2.5 Genomic DNA Isolation

Bacterial gDNA was isolated as protocol developed by Sambrook and Russel, 2001 (Fig. 7).

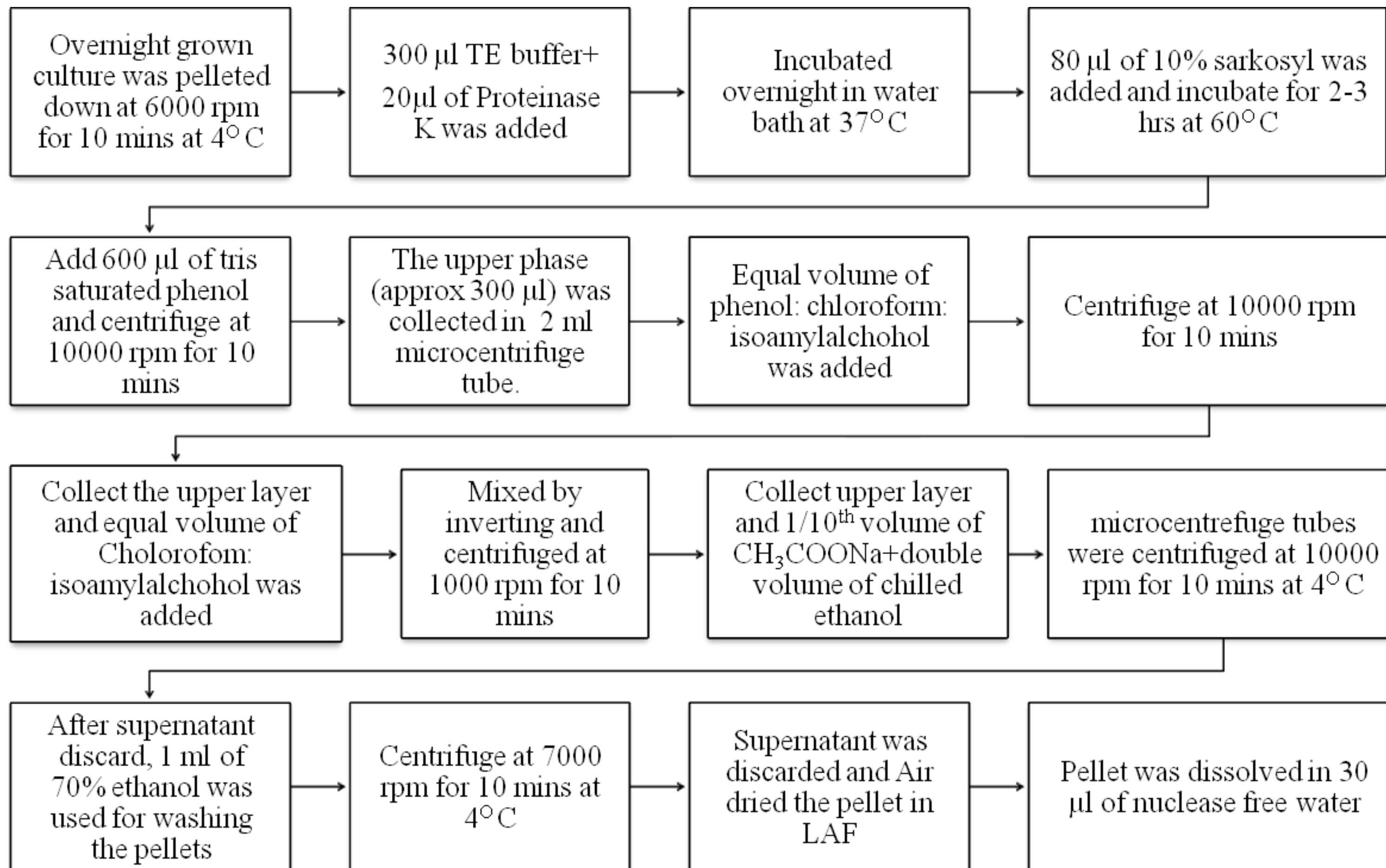

Fig. 7 Flow diagram for genomic DNA isolation

3.2.6 Agarose gel electrophoresis

gDNA quality was analyzed on EtBr stained agarose gel (0.8%)

Gel preparation

Components	Amount
Agarose	0.8 gm
1X TAE buffer	100 ml.
In a microwave, the mixture was boiled in oven and after cooled down to 45-50°C	
EtBr	1µl

In a gel casting tray, the mixture was poured and wait until it get solidified. After solidification the gel, gDNA along with loading dye was loaded and electrophoresed for 45 mins at 85V using PowerPac 300 (Bio-Rad).By the endof electrophoresis, visualization of gel was carried out in Biorad Gel Doc XR+ and image of the representative gel was being captured.

3.2.7 Quantification of the DNA

- OD_{260} and OD_{280} (optical density) of the isolated DNA was measured in UV-Vis spectrophotometer. The ratio of optical densities (OD_{260}/ OD_{280}) was calculated in order to examine the purity of the isolated genetic material.
- The DNA concentration was calculated using a mathematical equation-

$$\text{DNA concentration} = 50 \times OD_{260} \times \text{Dilution factor}$$

3.2.8 16S rRNA gene amplification

In a thermal cycler, 16S rRNA gene amplification was amplified by using primers reported by Kumar et al., 2014, Behera et al., 2017 (UFF2 5′-GTTGATCATGGCTCAG-3′ and URF2 5′-GGTTCACTTGTTACGACTT-3′). The composition of master mixture is shown in Table 2

Table 2. PCR master mixture composition

Master Mix	Quantity (µl)
dH_20	13.3
10X PCR buffer	2.5
25mM $MgCl_2$	1
Forward primer	2
Reverse primer	2
10mM dNTPS	1
Taq DNA polymerase	0.2
Template	3
Total	**25**

The PCR program for 16S rRNA gene amplification was similar to the program designed by Behera et al., 2017.Amplified PCR products were checked in 2% agarose gel containing EtBr. The gel was electrophoresed at 80 V for 40 mins. 100bp DNA ladder was used for molecular weight standard. The gel was observed in UV transilluminator and representative image was captured in Biorad Gel Doc XR+.

3.2.9 DNA sequencing and NCBI submission

Sequencing of the amplified products were outsourced at agrigenome, Kochi, India. DNA Bazer software v0.7.0, was used for contig preperation. Contigs of each isolates were kept in separate FASTA files and were BLAST analyzed in blastn server (http://www.ncbi.nlm.nih.gov\BLAST). Using NCBI Bankit program the nucleotide sequence was submitted and accession number was generated.

3.2.10 Phylogenetic analysis

In the present research study, three different phylogenetic trees were constructed. First phylogenetic tree was generated for authentication of 16S rRNA based identification of the target *Klebsiella* strains. FASTA sequences of 16S rRNA of different *Klebsiella* spp. were retrieved from NCBI Genbank server. Using CLUSTAL- W algorithm of MEGA 6 software, K1 gene sequence was aligned with other *Klebsiella* species and as an outgroup, *Rickettsia conorii* was used. MEGA 6

program was employed with highest Bootstrapping of 10,000 replications. Maximum-likelihood algorithms were employed to establish the phylogenetic tree (Tamura et al., 2013).

The second phylogenetic clustering was established to provide an insight into the possible way of transmission of the strains across the geographical locations and enable us to propose a probability of virulence across regions. The sequences alignment of the isolated strains *viz.*K1- K10 was performed using CLUSTAL W algorithm. For the generation of Phylogenetic tree, Maximum-likelihood algorithm was applied with highest bootstrapping value at 10,000 replications.

In the third Phylogenetic tree, the isolated *K. pneumoniae* strains of the present study were aligned with *K. pneumoniae* strains reported globally retrieved from NCBI Genbank program (Table 3). Alignment of the sequence was done in CLUSTAL W algorithm of MEGA 6 software. The phylogentic tree was constructed with highest bootstrap value (10000 replications). Maximum-likelihood algorithm was employed for the generation of phylogenetic tree.

Table 3. GeneBank Accession Numbers of *Klebsiella pneumoniae*16S rRNA gene sequences

Strain	Geographic Origin	Accession Number	Isolation source	Sequence Source
K1	Burdwan, India	KY003130	Fish	Present study
K2	Burdwan, India	KU612260	Fish	Present study
K3	Burdwan, India	KX010115	Fish	Present study
K4	24 Pargana North, India	KX010116	Fish	Present study
K5	24 Pargana North, India	KX170832	Fish	Present study
K6	Burdwan, India	MF680432	Fish	Present study
K7	Nadia, India	MF680483	Fish	Present study
K8	24 Pargana North, India	MF680516	Fish	Present study
K9	Nadia, India	MF680539	Fish	Present study
K10	Nadia, India	MF680540	Fish	Present study
MAA	India	JQ701742	--	NCBI
FCC7	China	JF772085.1	Field-collected adult gut	NCBI
LSRC119	China	JF772079	*Bactrocera dorsalis*	NCBI
LRC61	China	JF772061	*Bactrocera dorsalis*	NCBI
CSMCRI-22	India	JQ665363	Seaweed	NCBI
F1-2-10	China	KX350022	Reed field	NCBI
njp9	Bangladesh	KU992686	Fish	NCBI
DP20B	Brazil	KJ560980	Fish	NCBI
TERI BD13	India	KM503154	Oil contaminated soil	NCBI
BG13	India	KJ522785	Fish	NCBI
A1	India	KC249934	Sewage	NCBI

			water	
SW-2	China	KU353691	Sewage	NCBI
LZ-5	China	JX283459	Sputum	NCBI
NY1	USA	GU377208	Municipal wastewater	NCBI
Kp 5-1	USA	FJ823263	Field insect	NCBI
JCM 1662	Japan	NR_113240	---	NCBI
JCM 1662	Japan	NR_112009	---	NCBI
FRM41	Bangladesh	KX233852	Giant Freshwater Prawn	NCBI
FRM6	Bangladesh	KX233848	Giant Freshwater Prawn	NCBI
PD19	Thailand	LC093517	Soil	NCBI
TR17	Thailand	AB647144	Glycerol contaminated soil	NCBI
PD10	Thailand	LC093514	Soil	NCBI
ABZ11	Malaysia	KX266892	Antarctic seawater	NCBI
NOAM-B2	Malaysia	KU593479	crude oil	NCBI
V1.1	Vietnam	KC213799	*Penaeus monodon*	NCBI
BW003	South-Africa	KU946990	River	NCBI
28	South-Africa	KJ742499	*Busseola fusca*	NCBI
402-2	Russia	AY114159	---	NCBI
KW	Pakistan	AB642256	---	NCBI
CC	Pakistan	AB642255	---	NCBI
B9A	Malaysia	KJ725227	Fermented milk product	

KP1	Sri Lanka	KT985366	Atmospheric particulate matter	NCBI
KOL03	Sri Lanka	KY967368	Petroleum contaminated wastewater	NCBI
B11C	Malaysia	KJ725232	Fermented milk product	NCBI
3	Indonesia	AB999796	Marine sediment	NCBI
NLEP98-0472	Canada	AF228920	Human urine	NCBI
B9B	Malaysia	KJ725228	Fermented milk product	NCBI
SW	Pakistan	AB641122	---	NCBI

3.2.11 PCR Ribotyping

3.2.11.1 Amplification of Inter transcribed spacer region

Genomic DNA was extracted as per the protocol described above at section 3.2.5. PCR ribotyping of different isolated strains was carried out by targeting the region conserved for 16S and 23S rRNA gene sequence. The primers ($5'$ -TTGTACACACCGCCCGTCA -$'3$ and $5'$-GGTACCTTAGATGTTTCAGTTC-$'3$) developed by Kostman et al., 1992, wasincorporated for amplification of inter transcribed spacers region. 50 µL of PCR mixture comprised of autoclaved water, 5µl of 10X Buffer (Sigma, USA), 10mM dNTPs, 25 mM $MgCl_2$ (GCC Biotech), each Forward and Reverse primer (10 pmol/ µL), 1 unit of Taq polymerase (Sigma, USA) and 4 µL of template. The thermal profile consisted of initial denaturation for 2 min at 95 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, final extension for 7 min at 72 °C. 5 µl of the amplified PCR products were mixed with 1 µl of loading buffer (Invitrogen) and were loaded in 2% agarose gel containing EtBr. The gel was run in 1X TAE buffer at a constant voltage of 100 V for 40 mins. A 100bp DNA ladder was used for molecular weight standard. Gel was visualized in UV transilluminator and representative picture was captured in Biorad Gel Doc XR+.

3.2.12 Antibiotic susceptibility test and Multiple Antibiotic resistances (MAR)

Each of the isolates was tested for susceptibility against 25 different antibiotics by the method as developed by Bauer et al., 1966. Loopfull of bacterial cultures was inoculated in BHI broth tubes and incubated overnight at 37°C. The concentration of the inoculums was adjusted to 0.5 McFarland turbidity (1.5×10^8 CFU/ml) by using a sterile TSB media (Frederick, 2015). 100 µl of the culture was spread on Muller Hinton agar plates. Paper antibiotic disc purchased from Hi-media, India were placed on the inoculated Muller Hilton agar media plates and incubated overnight. After

overnight incubation, qualitative susceptibility results for each isolates against each antibioticswere noted down in terms of resistant and sensitiveas described by Wayne, 2011; Reller et al., 2009. The antibiotics discs used are polymyxin B, streptomycin, cefepime, chloramphenicol, erythromycin, ciprofloxacin, trimethoprim, ofloxacin, ceftazidime, netilmicinsulphate, tetracycline, piperacillin, colistin, imipenem, nitrofurantoin, tobramycin, nalidixic acid,doxycycline, ampicillin, rifampicin, gentamicin, cefixime, fosfomycin, dicloxacillin (Das et al., 2018).

After determining the antibiotic susceptibility of different isolate, MARindex value was calculated as formula provided by Sarter et al., 2007 (Fig. 8).

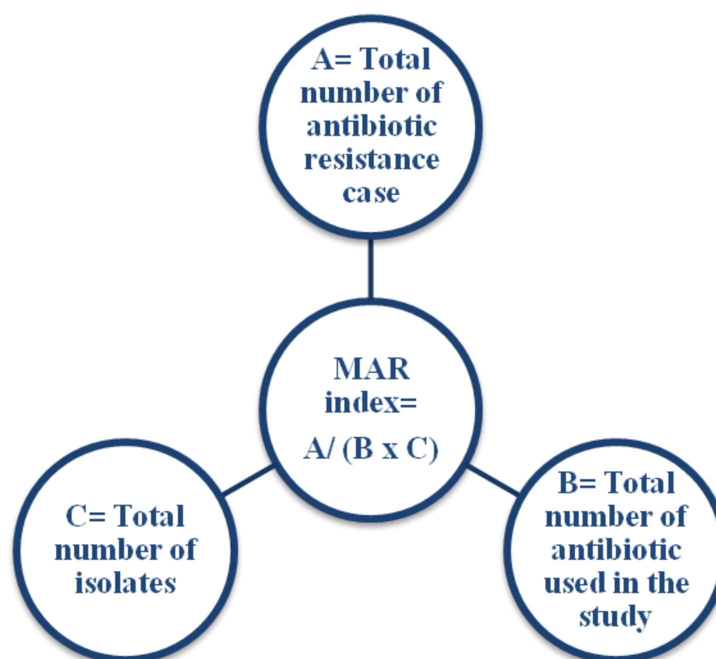

Fig. 8 Formula for determination of MAR value

3.2.13 Solid hemolysin test and liquid hemolysin assay

Hemolysis involves Red blood cells (R.B.Cs) breakdown by bacteria. Solid blood hemolysin assay was performed on 5% (W/V) blood agar plates as described by Gerhardt, 1994.

- Bacterial colonies were inoculated and incubated in BHI broth at 37° C for.24 hours.
- Simultaneously, 100 ml of blood agar (Hi-media) was autoclaved at 121°C at 15 psi for 15 mins.
- When the media cooled down to 45- 50 ^{o} C, 5 ml defibrinated sterile sheep blood was added to the media and stirred continuously by avoiding the formation of bubbles.
- The blood agar was poured into sterile petri plates and kept overnight to check for contamination.
- Contamination free plates were streaked with loop full of bacterial culture and incubated 48 hours at 37°C to check the zone of hemolysin.

Liquid hemolysin assay property of strains was studied using protocol developed by Deshpande and Khan, 1999.

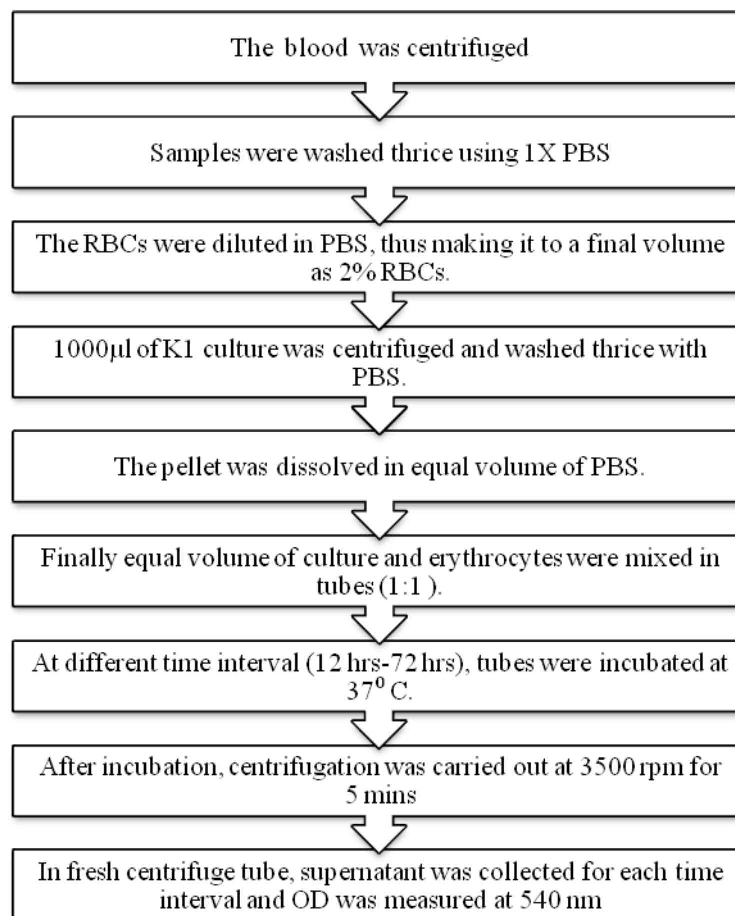

Fig. 9 Flow diagram describing steps for Liquid hemolysin assay

3.2.14 Fish Acclimatization for Experimental Challenge

From a local hatchery of North 24 parganas, healthy and infection free *L. rohita* juveniles (20.0 ± 3 g) were procured. The fishes were packed in aerated bags for brining to laboratory. After arrival of fishes, potassium permanganate dip treatment (2- 3 mins) was performed for the surface sterilization of the fishes. Acclimatization of fishes was carried out in well aerated 200 L FRP tanks. Everyday approximately 20% water from the tanks were exchanged and once weekly 100% of the water was exchanged. At 2% body weight of fishes, they were fed twice with pelleted feed. The Water qualities of the tanks were maintained using aerators and temperature controllers. During the experiment basic physiochemical parameters of water were measured weekly. The recorded water temperature varies from 26 ±2°C, the pH was maintained within 7.5- 8.0 and dissolved oxygen was maintained at 6 ±1.2 mg/L.

3.2.15 Determination of LD_{50} of *K. pneumoniae* in *Labeo rohita*

LD_{50} study was conducted to find out the number of *Klebsiella pneumoniae* colonies essential for 50% mortality of *L. rohita* population (Fig. 10). The experiment for determining the LD_{50}value was performed in triplicate (Control- 3 tanks and experiment- 24 tanks). To satisfy Koch's postulate, after determination of LD_{50} the bacteria was re-isolated from the internal tissues and reconfirmed by growing it on *Klebsiella* specific agar plate.

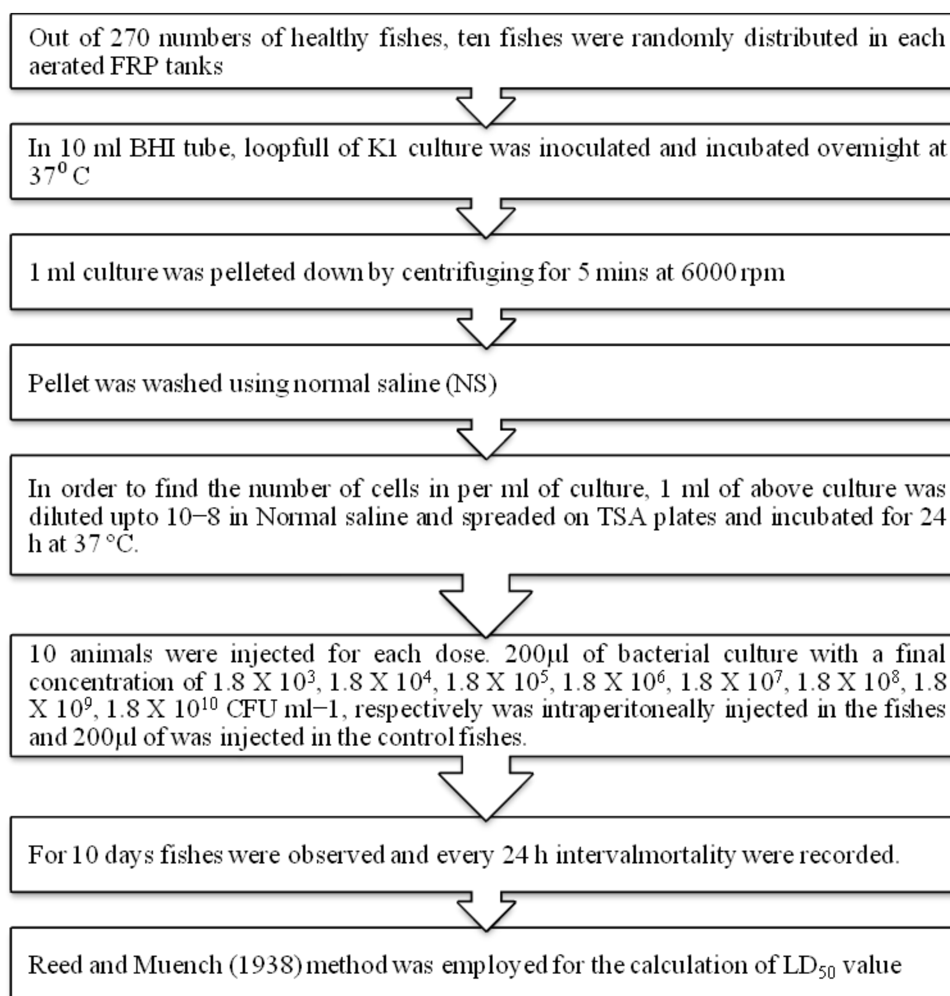

Fig. 10 Schematic representation of steps for LD_{50} determination

3.2.16 Histopathology

At the end of experimental time period, the external lesions on *Labeo rohita* fishes were observed. Alcohol was used for cleaning moribund fishes and Clove oil (Merck, Germany) (50µl/l) was used as anesthetizing agent. By using a scalpel and forcep, kidney and liver tissues were excised from the diseased fish samples. The tissues were anatomized (1- 2 mm) by using a sharp scalpel (Das et al., 2018).

A. Tissue preservation

- Neutral buffered formalin- 24hrs
- Washing under tap water- 2-3 hrs

- After washing, the tissues were stored in 70% alcohol and were processed further.

B. Tissue processing

The dissected tissues were placed in embedding cassettes (Hi-media) and were processed by keeping the blocks two times in 70% ethanol for 60 mins each. Further, the blocks were processed in 90% ethanol for two times for 60 mins each. Then the blocks were incubated in 100% ethanol for three times at a time interval of 60 mins. Further the blocks were incubated for 5 mins in xylene and then it was transferred to jar containing 50% xylene + 50 % paraffin for 60 mins. After incubation the blocks were processed in paraffin (58°C) for three times at a time interval of 60 mins.

C. Block preparation

After tissue processing, the tissues were placed on embedding molds at the desired position and were covered with Embedding Spare ''O'' Rings (Hi- media). The molds were kept on ice plate and wax (58°C) was poured in the mold. The molds were kept overnight at -4° C. After overnight incubation, the molds were removed and the rings having solidified wax were trimmed in microtome (Leica EG 1140H, Germany) at 10µm thickness. After trimming, the rings were again kept overnight at 4°C.

D. Slide preparation

Water bath was set at 58° C and glass slide were cleaned with 100% ethanol. The glass slides were mounted with egg albumin (Hi- media) and were kept to air dry. Further, the blocks were sectioned using a microtome (Leica EG 1140H, Germany) at 5 µm thickness. The paraffin ribbons were stretched on water bath and were mounted on the glass sides. The slides were kept overnight at 58-60°C in an incubator.

E. Staining of the slides

The staining was carried out with haematoxylin and eosin stain (Luna, 1968)

- Xylene I
- Xylene II
- 100% EtOH
- 90% EtOH
- 70% EtOH
- 50% EtOH
- 30% EtOH
- Harris' Hematoxylin
- Harris' Hematoxylin
- Tap water
- Acid alcohol- 3 mins
- Tap water- 5 mins
- Scotts tap water- 3 mins
- Tap water- 5 mins
- Eosin- 5-10 seconds
- 100% ethanol- 8mins
- 100% ethanol- 8mins
- Xylene I- 8mins
- Xylene II- 8mins

F. Preparation of permanent slides

Slides were mounted with DPX and coverslip was placed on the slide and air dried for 2-3 hrs. The slides were observed under light microscope (Nikon eclipse CI, N600) and images were captured (Nikon- DS12).

3.2.17 String Test for Hypermucoviscosity

Bacterial strain can be defined as hypermucoviscous, only if the bacterial colony produces a viscous string of >5 mm in length after stretching it with a loop (Fang et al., 2004).

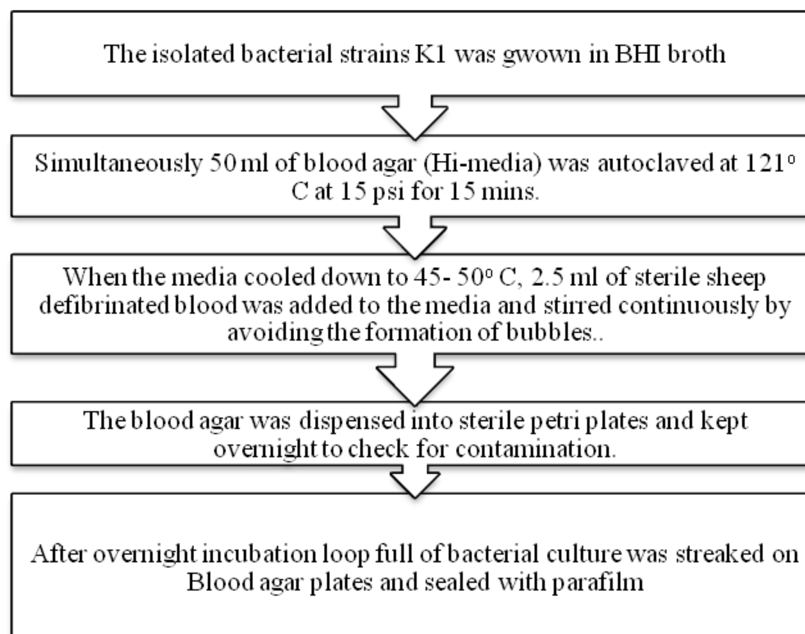

Fig. 11 Schematic representation of Hypermucoviscosity test

3.2.18 Detection of virulence genes in *Klebsiella pneumoniae* isolates

Presently, twelve virulent genes were screened of through PCR to characterize the isolates virulence property. The targeted virulent genes of the isolates include *fimA, fimH, entB, ureA*, *ugeA*, *wabG, ecpRAB, rmpA, magA, alls, kfu* and *mrkA*. Components for PCR master mixture were similar as Table. 2. Primers along with their annealing temperature are provided in table 4.The PCR profile was similar as shown in section 3.2.8. 2% agarose gel containing EtBr was used to verify the amplified genes. PCR products were visualized in. A 100bp DNA ladder was used for molecular weight standard. The gel was visualized in UV transilluminator. The representative image was captured in Biorad Gel Doc XR+.

Table 4. Primers used for detection of virulent genes

Sl. No.	Virulence factor or genes (product)	Primer sequence	Anneling temperature (°C)	Amplicon size (bp)	References
1.	*fimH* (fimbrial)	fimH-F5′TGGTGGTCGACCTCTCCACGCAGATTTTTGCC-3′ fimA-R 5′ TCAGCTGAACGCCTATCCCCTGCGCCGGCGAGGCGG-3′	62	575	Catalán-Nájera et al., 2017
2.	*fimA* (fimbrial)	fimA-F 5′- CGGACGGTACGCTGTATTTT-3′ fimA-R 5′- GCTTCGGCGTTGTCTTTATC-3′	62	438	Catalán-Nájera et al., 2017
3.	*mrkA* (fimbrial)	mrkA-F 5′-CGGTAAAGTTACCGACGTATCTTGTACTG-3′ mrkA-R 5′-GCTGTTAACCACACCGGTGGTAAC-3′	62	597	Catalán-Nájera et al., 2017
4.	*entB* (enterobactin)	entB-F 5′- GATGAAGACGATACCGTGC-3′ entB-R 5′- ACCGAATCCAGACCGTAGTC-3′	55	391	Catalán-Nájera et al., 2017
5.	*urea* (urease)	ureA-F 5′-GCTGACTTAAGAGAACGTTATG-3′ ureA-R 5′- GATCATGGCGCTACCTYA-3′	55	333	Catalán-Nájera et al., 2017
6.	*uge*	uge-F 5′- GATCATCCGGTCTCCCTGTA-3′ uge-R 5′- TCTTCACGCCTTCCTTCACT-3′	51	538	Catalán-Nájera et al., 2017
7.	*wabG*	wabG-F 5′- CGGACTGGCAGATCCATATC-3′ wabG-R 5′- ACCATCGGCCATTTGATAGA-3′	53	680	Catalán-Nájera et al., 2017
8.	*ecpRAB* (fimbrial)	ecpF5′- CCTATGTAATTAATGGCAGGTTT-3′ G511 5′-GCTGTTCATAAAGGATGAAATATC-3′	62	1025	Catalán-Nájera et al., 2017
9	*magA*	magAF- 5′- GGTGCTCTTTACATCATTGC-3′ magAR- 5′- GCAATGGCCATTTGCGTTAG-3′	53	1282	Fang et al., 2004
10	*rmpA*	rmpAF-5′- ACTGGGCTACCTCTGCTTCA-3′ rmpAR- 5′- CTTGCATGAGCCATCTTTCA-3′	50	535	Nadasy et al., 2007
11	*alls*	Alls F 5′- CCGAAACATTACGCACCTTT-3′ Alls R 5′-ATCACGAAGAGCCAGGTCAC-3′	50	508	Yu et al., 2008
12	*kfu*	Kfu F 5′- ATAGTAGGCGAGCACCGAGA-3′ Kfu R 5′-AGAACCTTCCTCGCTGAACA-3′	50	520	Yu et al., 2008

3.2.19 Sequencing and NCBI Submission

Sequencing was carried out at agrigenome, Kochi, India. 20µl of each PCR amplified products were sent for sequencing. The reverse and forward sequence for each samples were aligned in DNA Bazer software version v0.7.0 and contigs were prepared. Contig of each amplified gene was analyzed using NCBI nucleotide BLAST (blastn) program (http://www.ncbi.nlm.nih.gov\BLAST). The sequences were submitted through NCBI Bankit program in NCBI genbank database and accession number for each amplified virulent gene was received.

3.2.20 Challenge test for Non- specific immune response and specific gene expression studies

3.2.20.1 Experimental design

Live, apparently healthy Rohu (*L. rohita*) juveniles weighing of about 25 ± 2.0 g) were procured from a local hatchery. The fishes were acclimatized under laboratory conditions for fifteen days.The fishes were fed two times daily at the rate of 2% of their body mass. Thefisheswere distributed randomly in 21 independent Fibreglass Reinforced Plastics (FRP) tanks. Experiment was carried out in triplicate. Total 210 fishes were equally distributed in 21 tanks with 10 fishesin each tanks (10 × 3 tank as control and 10×18 tanks for experimental challenges). During the experiment D.O, temperature and pH values were 7 ± 1 mg/ l, 28 ± 2.0^{o}C and 6.9 ± 0.62 respectively. The challenge group fishes were injected with 200 µl of the bacterial culture dissolved in PBS with the determined LD_{50} value and control group was injected with 200 µl of PBS only. The fishes were kept under surveillance.

3.2.21 Non Specific immune response

3.2.21.1 Sampling and blood collection

The fishes were collected at an interval of 12- 72 hours post infection (hpi). The fishes were anaesthetized by bath treatment in MS-222 solution (Hi media) (150 ppm). Blood were drawn from the caudal vein with 24 gauge needle and 2 ml syringe. The blood were distributed into two different aliquots set, one with heparin (50 I.U/ml of blood) and the other tube without anticoagulant was kept to clot at RT for 30 mins and the tubes were kept at 4° C for 2-3 hrs. The tubes with clotted blood were centrifuged for 10 mins at 3000 rpm. The supernatant containing serum was collected and transferred into a fresh microcentrifuge 1.5 ml tubes. The collected serum samples were aliquoted and stored at − 20° C for further use.

3.2.21.2 Respiratory burst activity

Respiratory burst activity was measured as protocol developed by Anderson and Siwicki, 1995).

Blank	Test
100 µl blood	100 µl blood
↓	↓
100 µl of PBS	100 µl of NBT
↓	↓
Incubated at 25°C for 30 minutes	Incubated at 25°C for 30 minutes
↓	↓
Take 50 µl of reaction mixture	Take 50 µl of reaction mixture
↓	↓
Add 1 ml of Dimethyl formamide	Add 1 ml of Dimethyl formamide
↓	↓
Centrifuge at 3000 rpm for 5 minutes	Centrifuge at 3000 rpm for 5 minutes
↓	↓
Measure OD at 540nm	Measure OD at 540nm

3.2.21.3 Myeloperoxidase activity (MPO)

In serum, the total MPO content present was calculatedby using protocol developed by Quade and Roth, 1997with slight modification. HBSS (Hanks Balanced Salt Solution) is made up of inorganic salts supplemented with glucose. The solution is supplied with phosphate buffer. So that while washing the cells or tissue, it will maintain the physiological pH and osmotic pressure.

Blank	Test
10 µl Serum ↓ 90 µl of HBSS (Sigma, USA) ↓ 35 µl PBS + 20 µl of H_2O_2 ↓ Incubate for 2 min at room temperature ↓ Add 35 µl of 4M H_2So_4 ↓ Measure OD at 540nm	10 µl Serum ↓ 90 µl of HBSS (Sigma, USA) ↓ 35 µl TMB + 20 µl of H_2O_2 ↓ Incubate for 2 min at room temperature ↓ 35 µl of 4M H_2So_4 ↓ Measure OD at 540nm

3.2.21.4 Total antiproteases activity

In serum, the total antiproteases activity was determined according to the protocol by Zuo and Woo, 1997 with slight modifications.

Blank	Test	Positive control	Negative control
10 µl Serum ↓	10 µl Serum ↓	100 µl trypsin (200ug/ ml in PBS) ↓	100 ul PBS ↓
100 µl PBS ↓	100µl trypsin (200ug/ ml in PBS) ↓	100 µl PBS ↓	Incubate for 30 min at 25°C ↓
Incubate for 30 min at 25°C ↓	Incubate for 30 min at 25°C ↓	Incubate for 30 min at 25°C ↓	Add 1ml of casein ↓
Add 1ml of casein ↓	Add 1ml of casein ↓	Add 1ml of casein ↓	Incubate for 30 min at 25°C ↓
Incubate for 30 min at 25°C ↓	Incubate for 30 min at 25°C ↓	Incubate for 30 min at 25°C ↓	Add 500 µl of 10% of trichloroacetic acid ↓
Add 500 µl of 10% of trichloroacetic acid ↓	Add 500 µl of 10% of trichloroacetic acid ↓	Add 500 µl of 10% of trichloroacetic acid ↓	Measure OD at 280 nm
Measure OD at 280 nm	Measure OD at 280 nm	Measure OD at 280 nm	

Calculation:

Reference value = (absorbance of positive control- absorbance of negative control)

Control value = (absorbance of test sample- absorbance of respective serum blank)

Percent inhibition = (reference value - control value) / Reference value X 100

3.2.21.5 Alpha-2 macroglobulin (α-2 M) activity

The α-2 M activity of in fish serum was determined by the Zuo and Woo, 1997 with partial modification.

Blank	Test	Positive control	Negative control
80 µl 50mM Tris-HCl ↓ 10 µl Serum ↓ 10 µl 20mM CaCl2 ↓ Incubate for 40 min at 25°C ↓ Add 1ml of BAPNA ↓ 90 µl 20mM CaCl2 ↓ Incubate for 20 min at 25°C ↓ Add 250 µl of 30% V/V Acetic acid ↓ Measure OD at 410nm	30 µl 50mM Tris-HCl ↓ 10 µl Serum ↓ Add 50 µl of 100 µg/ml trypsin ↓ 10 µl 20mM CaCl2 ↓ Incubate for 40 min at 25°C ↓ Add 1ml of BAPNA ↓ 90 µl 20mM CaCl2 ↓ Incubate for 20 min at 25°C ↓ Add 250 µl of 30% V/V Acetic acid ↓ Measure OD at 410nm	40 µl 50mM Tris-HCl ↓ Add 50 µl of 100 µg/ml trypsin ↓ 10 µl 20mM $CaCl_2$ ↓ Incubate for 40 min at 25°C ↓ Add 1ml of BAPNA ↓ 90 µl 20mM $CaCl_2$ ↓ Incubate for 20 min at 25°C ↓ Add 250 µl of 30% V/V Acetic acid ↓ Measure OD at 410nm	90 µl 50mM Tris-HCl ↓ 10 µl 20mM $CaCl_2$ ↓ Incubate for 40 min at 25°C ↓ Add 1ml of BAPNA ↓ 90 µl 20mM $CaCl_2$ ↓ Incubate for 20 min at 25°C ↓ Add 250 µl of 30% V/V Acetic acid ↓ Measure OD at 410nm

The Percentage of trypsin inhibition was calculated by the following formula.

Reference value = (absorbance of positive control- absorbance of negative control)

Control value = (absorbance of test sample- absorbance of respective serum blank)

Percent inhibition = (reference value - control value) / Reference value X 100

3.2.21.6 Lysozyme activity

The lysozyme activity level was measured by using the protocol developed by Sankaran and Gurnani, 1972 with few modifications.

Standard	Test
Lysozyme solution of 0µg/ml, 5 µg/ ml, 10 µg/ ml, 15 µg/ ml, 20µg/ ml and 25 µg/ ml concentration in 0.02 M sodium acetate (pH 5.5)	25 µl Serum
↓	↓
Add 125 ul of *Micrococcus lysodeikticus*	Add 125 ul of *Micrococcus lysodeikticus*
↓	↓
Take immediate OD at 450nm and incubate plate at 24°C for I hr	Incubate at 24°C for 1 hr
↓	↓
After incubation, OD was recorded at 450 nm	Measure OD at 450 nm

- After getting the OD, Subtract OD= Initial OD- Final OD,(Initial OD= Conc. At 0 µg/ml)
- A graph standard curve was prepared by using the lysozyme concentration on X- axis and subtracted OD on Y –axis.
- The concentration of the serum lysozyme was calculated with the help of the above graph

3.2.22 Gene expression study

3.2.22.1 Sample collection of RNA isolation

After blood collection, the fishes were cleaned and dissected. Tissue samples of liver, Kidney and Muscle were collected from both the infected and the control group fishes in RNA Later solution (Sigma, USA).

3.2.22.2 RNA isolation

Total RNA was extracted from the liver, kidney and muscle tissues using Trizol method (Chomczynski et al., 1995)

- 100mg of tissues were homogenized in 1 ml Trizol by using a glass homogenize in cold condition (Sigma, USA).
- The homogenate was shifted to fresh 1.5 ml RNAse free centrifuge tube.
- 200µl of ice cold chloroform was added and vortexed for 2 mins.
- The tube was centrifuged at 14000 rpm for 10 mins at 4°C.
- 500ul of supernatant was taken out in fresh microcentrifuge tube and equal volume of ice cold isopropanol was added.
- The tube was kept at -20°C for overnight.
- After overnight incubation the tube was centrifuges at 14000 for 10 mins at 4° C
- The supernatant was discarded and pellet was washed with 75% ice cold ethanol.
- The tube was centrifuged at 7000 rpm for 10 mins at 4°C.
- The pellet was air dried for 15- 20 mins in a laminar air flow.
- 40µl of DEPC treated nuclease free water was added to dissolve the pellet

- 5 µl of DNA sample was mixed 1 µl of DNA loading dye and loaded into each well of 2% agarose gel. The unit was connected with power pack and electrophoresis was carried out at 85 V for 45 mins. After completion of the run, the gel was visualized in Biorad Gel Doc XR+ and image of the representative gel was being captured.

3.2.22.3 DNAse treatment

The isolated RNA was purified using Ribopure RNA purification kit (AM1925, ThermoFisher scientific) by the following protocol-

- $1/9^{th}$ volume of 10X DNAse buffer and 4µl of DNAse I was mixed with the isolated RNA
- The tube was incubated for 30 mins at 37^oC
- DNA inactivation reagent was added at a volume of 20% of the volume of the RNA
- The tube was vortexed vigorously
- The tube was incubated for 2 mins at room temperature
- Then the tube was centrifuged at 10000 rpm for 1 mins
- The supernatant was collected and transferred to a fresh RNAse free 1.5 ml microcentrifuge tube and stored at -80^oC for further use.

3.2.22.4 Quantification of the RNA

- The optical density (OD) was calculated at both wavelengths 260 nm and 280 nm. The ratio of optical densities (OD_{260} / OD_{280}) was calculated.
- The concentration of the RNA was calculated using a mathematical equation.

$$\text{Concentration of DNA} = 40 \times OD_{260} \times \text{Dilution factor}$$

3.2.22.5 cDNA Synthesis

cDNA were synthesized by using 5µg of RNA isolated from different fish tissue of both control and infected fishes following the manufacture protocol (cDNA synthesis kit, K1632- Thermo).

Reagents	Quantity (µl)
Oligo dT	1
Random Hexamer	1
Nuclease free water	5
RNA	5

- The reagents were mixed by vortexing in a 0.2 ml tube and incubated at 65°C for 5 mins.

  After incubation, the following reagents were added to it

Reagents	Quantity (µl)
5X reaction buffer	4
10mM dNTPs	2
Ribolock	1
Revertaid	1

- The tube was vortexed and centrifuged for a short spin
- cDNA synthesis was carried out in a thermal cycler. The thermal profile consisted of 25° C for 5 mins followed by 42°C for 60 mins and 70°C for 5 mins. The cDNA was stored at -20° C for further use.

3.2.22.6 Quantitative Real Time PCR (qRT-PCR)

Primer sequences of C3, IL6 and IL-1β were obtained from previous study (Table 5). β- actin was used as the housekeeping gene. Quantitative PCR (qPCR) was

performed using FastStart Essential DNA Green Master (Roche, Germany) in Light Cycler 96 (Roche, Germany). Briefly, 2 μl of synthesized cDNA was used as a template in a total reaction mixture containing 10μl of 2XLight cycler SYBR green I mix, 1μl of each forward and reverse primer (5 pmole) (Table 1), and 6μl of H_2O provided in the kit.The real time PCR (qPCR) program consisted of pre incubation at 95° C followed by amplification of 40 cycle at 95°C for 10 s, annealing temperature for respective genes (Table 5) for 10 s, and 72 °C for 10 s. Specificity of qPCR was verified by melt curve analysis at a temperature of 95° C for 5 s, 65° C for 1 min, and 97° C for 1 min. The samples were cooled down at 40° C for 10 s.TheCt values were calculated using Light Cycler 96 SW 1.1 and the data were exported. By calculating the average of each Ct for the triplicate sample, N-fold differential expression was calculated (Livak and Schmittgen 2001). The Ct value of the gene for each cDNA was subtracted from its respective Ct value of housekeeping gene (β-actin) to get the ΔCt value. Averages of the ΔCt values were obtained from the triplicate data of each for each time period. Further, the ΔΔCt was calculated by subtracting the ΔCt of the samples from the ΔCtvalue of the calibrator. Fold difference was calculated as $2^{-\Delta\Delta Ct}$. Mean fold difference was calculated and represented as ±standard error.

Table 5. Primers used for immune gene expression study

Target Gene	Primer name	Primer	Annealing temperature (^{o}C)	Reference
β- actin	β-actin FW	5′-AGACCACCTTCAACTCCATCATG-3′	60	Basu et al., 2015
	β actin RW	5′-TCCGATCCAGACAGAGTATTTACGC-3′		
Complement C3	C3 F	5′-CCCTGGACAGCATTATCACTC-3′	60	Huttenhuis et al., 2006
	C3 R	5′-GATGGTCGCCTGTGTGGT-3′		
Interleukin 1β	IL-1β F	5′-ATCTTGGAGAATGTGATCGAAGAG-3′	54	Giri et al., 2015
	IL-1β R	5′-GATACGTTTTTGATCCTCAAGTGTGAAG-3′		
Interleukin-6	IL-6F	5′-GGACCGCTTTGAAACTCT-3′	60	Kjoglum et al., 2006
	IL-6R	5′-GCTCCCTGTAACGCTTGT-3′		

3.2.23 Statistical analysis

The significance of difference was statistically analyzed using paired t- Test followed by using MS Excel software, and $P < 0.05$ indicated statistical significance. All data were expressed as mean ± standard error of the mean (SEM).