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Identification and Artificial Infestation of *Staphylococcus aureus* Isolated from Diseased Fish, *Labeo rohita*

Ashaduzzaman, Rezina Laz and Biswanath Sikdar

Abstract
The present study was conducted on diseased fish, *Labeo rohita* collected from in Rajshahi city corporation area. Bacterial isolates, $BSLR_1$, $BSLR_2$ and $BSLR_3$ were identified. The isolates showed round/spherical shape with pale yellow and yellowish pigmentation. All bacterial isolates were gram positive and showed negative reactions in oxidase, potassium hydroxide, Simmon citrate, MacConkey, methyl red and urease tests as well as positive reactions in catalase test. $BSLR_3$ showed positive in triple sugar iron test while $BSLR_1$ and $BSLR_2$ were negative. $BSLR_2$ were positive in sulphur indole motility test but $BSLR_1$ and $BSLR_3$ showed negative. $BSLR_2$ and $BSLR_3$ exhibited positive reactions in kligler iron test but $BSLR_1$ was negative. Antibiotic sensitivity test was performed in this study. All bacterial isolates showed sensitivity to Erythromycin, Azithromycin, Gentamycin and resistant as well as intermediate to Oxytetracycline and Neomycin respectively. In case of Doxycycline, $BSLR_1$ showed intermediate but $BSLR_2$ and $BSLR_3$ were sensitive. $BSLR_3$ showed intermediate to cefotaxime but others exhibited sensitive. $BSLR_1$ showed intermediate to Sulfonamide whereas others were sensitive. Three strains of *Staphylococcus aureus* was confirmed by Molecular identification. In aartificial infestation, all isolates showed no infestation at 100µl suspensions but at 300µl, infestations were found in all fish aquaria. All isolates showed both infestation and non-infestation at 200µl suspensions.

Keywords: Isolation, molecular characterization, artificial infestation, *Staphylococcus aureus*, *Labeo rohita*

Introduction
Fish is a vital source of food for people and contributes about 60% of the world's supply of protein. Sixty percent of the developing countries derive 30% of the annual protein from fish [1]. It is the most important source of high quality protein, providing approximately 16% of the animal protein consumed by the world's population [2]. Fish and fish products are the most important source of protein and it is estimated that more than 30 % of fish for human consumption comes from aquaculture [3]. Bangladesh has highly diversified fisheries resources and uniquely endowed with the diverse of very rich and extensive inland and marine fishery resources in the forms of ponds, natural depressions (haors and beels), lakes, canals, rivers and estuaries covering an area of 4.56 million hactar [4, 5, 6]. Fish is the second most important agricultural product in Bangladesh. Millions of people in Bangladesh lead their livelihoods directly or indirectly on fish farming and marketing [7]. The farming and consumption of fish and fish products therefore have important implications for Bengali nation and it's food demand. The people of Bangladesh are therefore popularly referred to as "Mache Bhate Bangali" or "fish and rice makes a Bengali" [8].
Fisheries is a diverse sector in Bangladesh but it's production is hampered and decreased due to the various kinds of diseases. Disease is the main restraining and limiting factors for the development of fisheries sector in Bangladesh. Fish disease is one of the furthermost frightening aspects [9]. A global estimate of disease losses to aquaculture estimated by World Bank in 1997 was in the range of US$ 3 billion per annum [10]. In spite of tremendous potential production, rural freshwater aquaculture of Bangladesh has been suffering from outbreak of diseases. It was reported the average economic loss of BDT 20,615/ha/year (equal to US$ 344) to rural freshwater fish farmers due to fish disease in Bangladesh [11]. Such loss affects the livelihood of poor people involved in the aquaculture sector. Therefore, disease is considered one of the important limiting factors in the reduction of fish production in Bangladesh.
The common fish diseases are epizootic ulcerative syndrome (EUS), tail and fin rot, bacterial gill rot, bacterial haemorrhagic septicaemia, columnaris disease, dropsy,

Staphylococcosis and streptococcosis in farmed fishes of Bangladesh [12]. However, fish is susceptible to a wide variety of bacterial pathogens, most of which are capable of causing disease [13]. Infectious diseases caused by bacteria have been recognized as a serious threat to aquaculture in Bangladesh. A range of aquatic diseases like *Staphylococci* or specific *Staphylococcus aureus* is one of the most virulent pathogenic bacteria and caused diseases in animals including fish. The presence of *Staphylococcus aureus* in marine environments caused diseases in skin, eye or ear of fish [14]. *Staphylococcus aureus* is considered to be the most important pathogen among staphylococci responsible for a variety of severe infections in human, fish and other animals [15]. The major habitats of *Staphylococcus aureus* are the skin and mucous membranes of human, fish and animals [16]. The infestation and infection process of *Staphylococcus aureus* involves colonization, local infection, systemic dissemination or sepsis, metastatic infections and toxinosis. *Staphylococcus aureus* produced a wide variety of virulence factors that are involved in pathogenesis. These factors are enterotoxins, adhesion proteins, toxic shock syndrome toxin (TSST), exfoliative toxin (ETA, ETB), pore-forming hemolysins, ADP-ribosylating toxin and proteases [17]. These toxins and enzymes are responsible for the lesions and wounds during the development of the infestation and infection in fish. Once *Staphylococcus aureus* penetrates the subcutaneous tissues and reaches the blood stream, it can infect almost any organ and tissue [18]. Molecular methods such as PCR using different DNA targets have been used successfully for the identification of *Staphylococci* at the species level [19]. The use of universal pathway genes and universal function genes whose nucleotide sequences are more conserved in bacteria as DNA targets for PCR amplification is becoming more and more frequent [20].

Staphylococcosis is associated with acute and chronic mortality in many aquaculture species [21]. The considerable diversity of *Staphylococci* bacteria associated with fish and caused serious damage as well as economic loss to the aquaculture. Aquaculture production in the country has been facing problems due to the outbreaks of disease and it imparted a noticeable effect to the fish production of Bangladesh. Therefore, present study was designed to conduct the isolation and identification of pathogenic bacterium, *Staphylococcus aureus* from diseased fish, *Labeo rohita* collected from Rajshahi city corporation areas.

Materials and Methods

Study Area

The study was conducted in the different ponds and fish markets of Rajshahi city corporation area, Rajshahi, Bangladesh (Fig. 1).

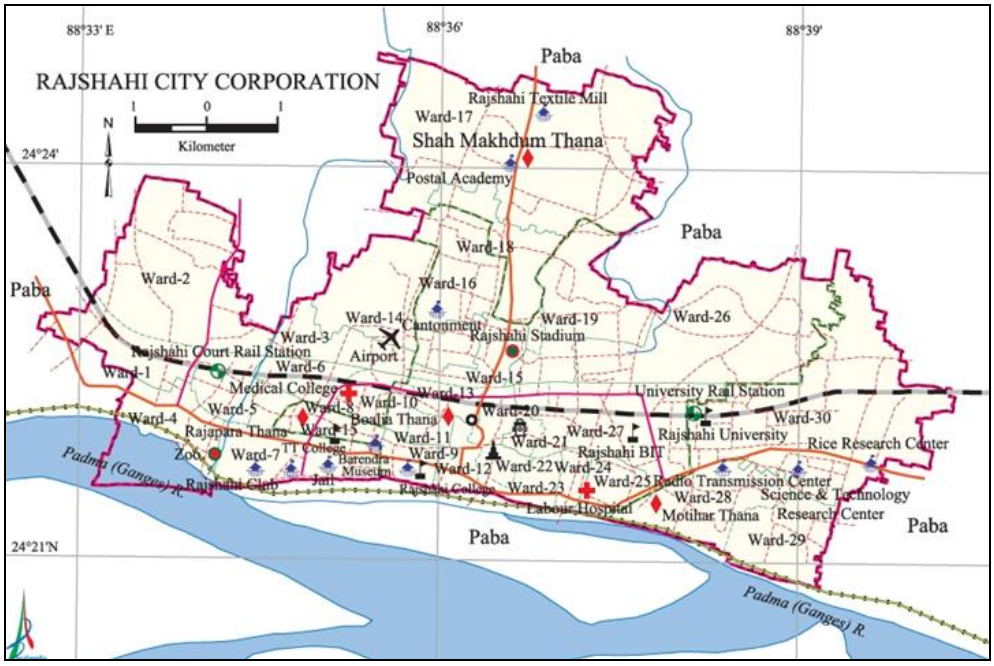


Fig 1: Map of the study area

Collection of Fish Samples

Diseased fish samples of *Labeo rohita* were collected from different ponds and markets of Rajshahi city corporation. The collected fish samples were packed in polythene bags and transferred to the laboratory for bacterial isolation and identification as well as bacteriological assays (Fig. 2).

Fig 2: Diseased fish samples collected from different ponds and markets of Rajshahi city corporation area.

Sample preparation, serial dilution and isolation of bacterial strains

The diseased portions of the fish bodies were scraped and one gram of each grinded fish flesh was suspended in 10mL of distilled water separately in three different test tubes and vortexes for 1 minute gently. It was allowed to settle down and 1 mL of suspension was inoculated into 100 mL of Bushnell Haas broth medium in a 250mL conical flask and placed in shaker for the enrichment of microbes.

Then the microbes were grown in the broth medium. At the same time the corresponding control groups were also maintained. Then it was subjected to serial dilution. One ml of 10^{-1} dilution was transferred again to another 9 ml of sterile distilled water in another test-tube. In such way serial dilution of the samples were made up to 10^{-4} (Fig. 3).

Then one ml of the diluted bacterial cell suspension was poured and spread over sterilized plates containing nutrient agar. The mixed bacterial cultures grew. One of single colonies was picked from each culture plate by wire loop and streaked on agar media plates for pure culture. Plates were incubated at 37°C for 24 hours. After several sub cultures, single pure colonies were grown but differing in morphological characteristics and were selected as well as used for further studies (Fig. 4).

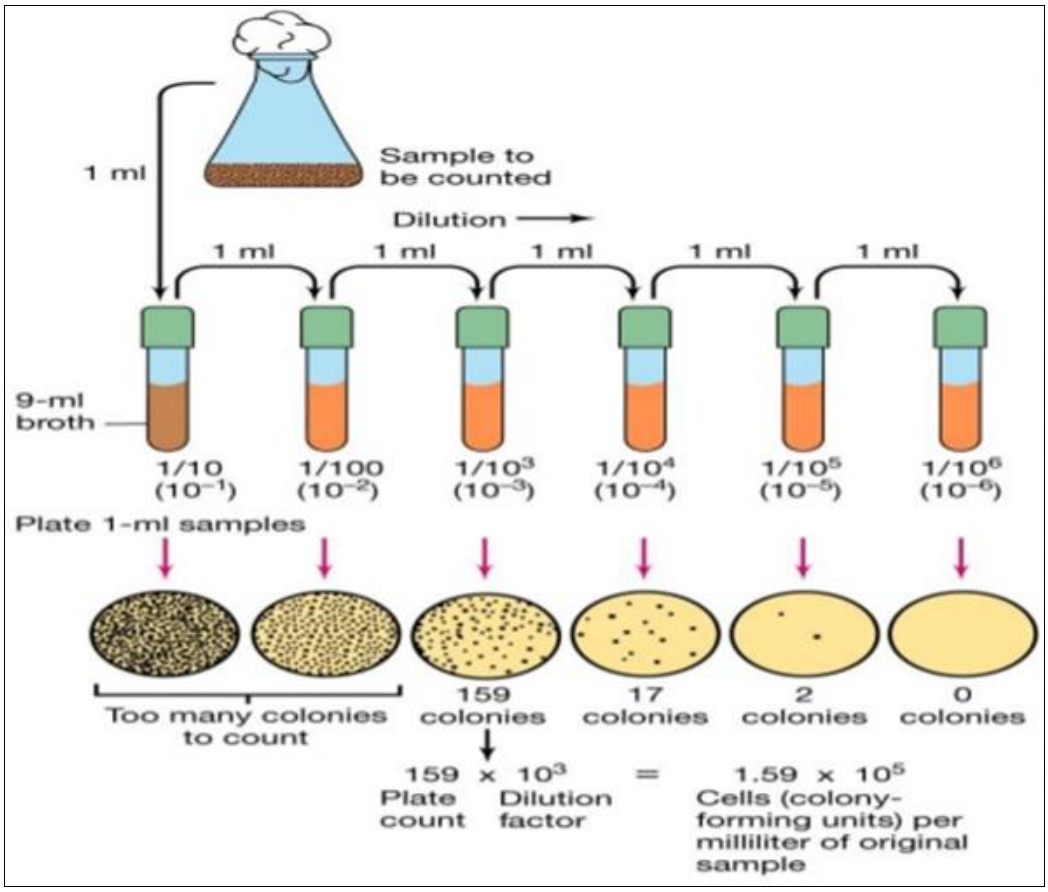


Fig 3: Serial Dilution

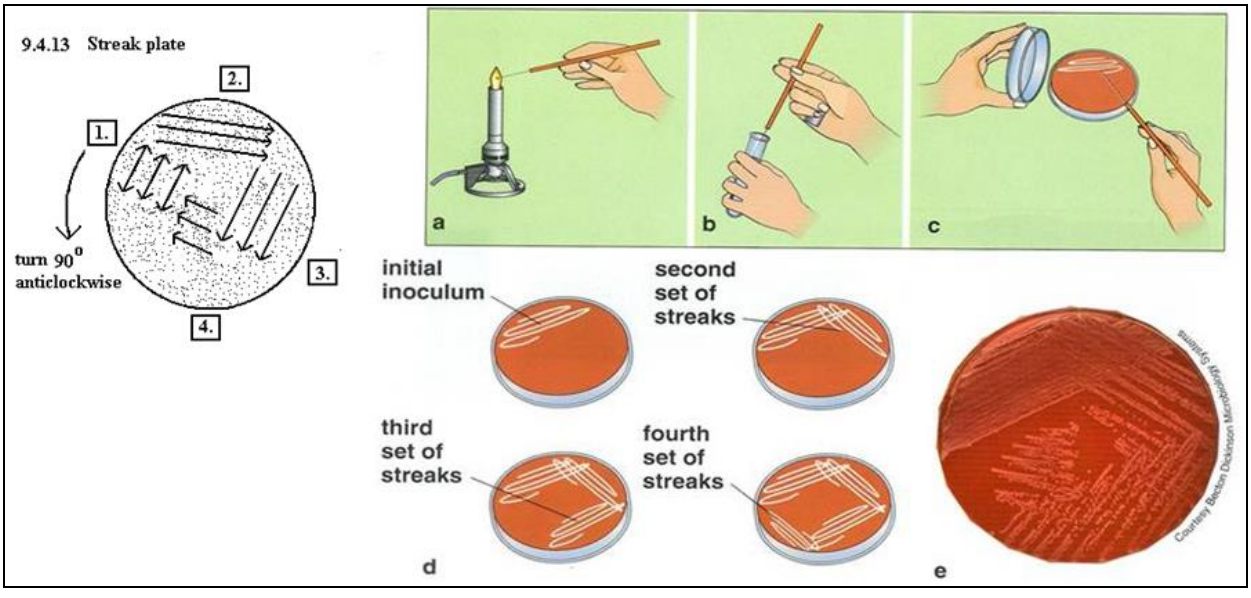


Fig 4: Procedure of streaking plates for the isolation of single bacterial colony

Procedure of bacterial identification

The single colony bacterial plates were marked as $BSLR_1$, $BSLR_2$ and $BSLR_3$, (Bacterial Sample from *Labeo rohita*) and subjected to the identification. Identification of the bacteria was conducted by some morphological, biochemical and molecular tests.

Morphological identification

Colony characteristics and cell morphology

The isolates were inoculated both on solid and liquid LB medium. Colony characteristics of the isolates on the medium were noted after 24 hours incubation. The growth patterns in both the liquid and solid medium were observed. The shape and size of the cells were observed by the microscope.

Gram's staining technique

A drop of sterilized distilled water was taken on the cleaned slide. A loopful bacterial suspension was transferred and heated to fix the sample to the slide. Then the smear was flooded with crystal violet and flooded with iodine solution as well as washed with alcohol for 5 seconds. The slides were covered with safranin and finally washed with water. The bacteria retained the crystal violet and the slides were examined under the microscope.

Biochemical identification

The purified *Staphylococcus aureus* isolates were identified through different biochemical tests (Oxidase, Catalase, KOH, Triple sugar iron, Sulfur indole motility, Simmon citrate, MacConkey agar, Kligler iron agar, Methyl red and Urea hydrolysis) [22].

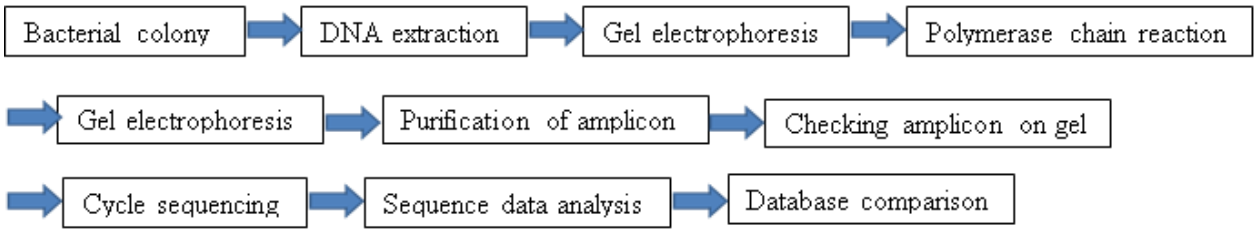
Antibiotic sensitivity test

The sensitivity of isolated bacteria to antibiotics was performed. Briefly, 200 µl of fresh broth culture of isolated bacteria was spread uniformly on a nutrient agar plate with a sterile glass spreader. The plates were air-dried for few minutes and then antibiotic discs were placed on inoculated nutrient agar plates, which were later incubated at 37°C for 24 hours. After incubation, the clear zones indicated the inhibition of growth of the isolated bacteria. Then, the zone was observed on the plate was measured with the help of mm scale.

Molecular identification

Molecular identification of the isolates, $BSLR_1$ $BSLR_2$ and $BSLR_3$ was conducted using a PCR for the species-specific fragment (Sa442) [23]. Genomic DNA was extracted using a GF-1 bacterial DNA extraction kit (Vivantis Technologies, Selangor DE, Malaysia) following the manufacturer's instructions for Gram positive bacteria. Bacterial isolates were identified using primer sequences of CGG TTA CCT TGT TAC GAC TT and AGA GTT TGA TCM TGG CTC AG.

Steps of Molecular identification



Artificial Infestation

After the proper identification of isolated bacterial species of $BSLR_1$, $BSLR_2$ and $BSLR_3$, the carp fish was artificially infested by those known bacterial strains. The pectoral, anal and caudal fins of carp fish were injected by 100 µl, 200 µl and 300 µl identified bacterial suspensions of $BSLR_1$, $BSLR_2$ and $BSLR_3$. Artificial infestation was conducted according to the growth factors of identified bacterial strains. These identified bacterial species have the common growth factors in temperature (35-40^{0}C) and p^H (6-8), were recorded regularly during the infestation period. The infestation appeared after 15 days. The infestation was recorded by observation of lesion, clinical appearance and mortality. Moribund fish were attended, observed and waited for the death. Immediately after the death, the fish were transferred to the laboratory for the observation of clinical signs and symptoms to confirm the pathogenicity by desired bacterial species (Fig. 5).

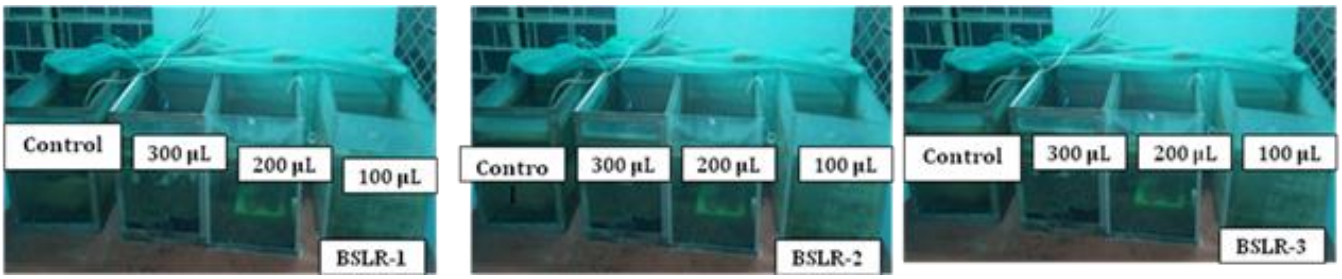


Fig 5: Procedure of artificial infestation on carp fish by identified known bacterial isolates of $BSLR_1$, $BSLR_2$ and $BSLR_3$.

Results

Single pure colony isolation and identification of bacterial strains

In the present study, three bacterial colonies namely $BSLR_1$, $BSLR_2$ and $BSLR_3$ were isolated successfully from *Labeo rohita* (Fig. 6-7). The bacteria were partially identified based on the color and colony morphology. Microscopic observations of the bacterial strains were done after gram staining and additional morphological test were conducted to identify the isolated bacteria and are presented in the Table 1-2 and Fig. 8.

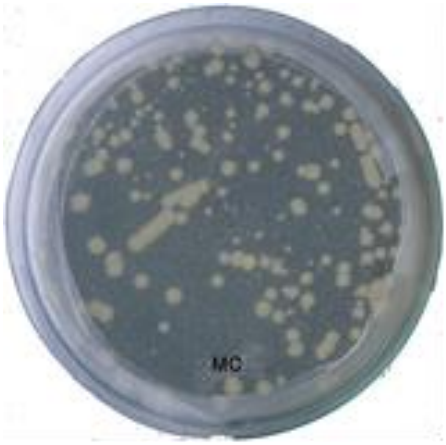


Fig 6: Primary or mother bacterial plate with different mixed colonies

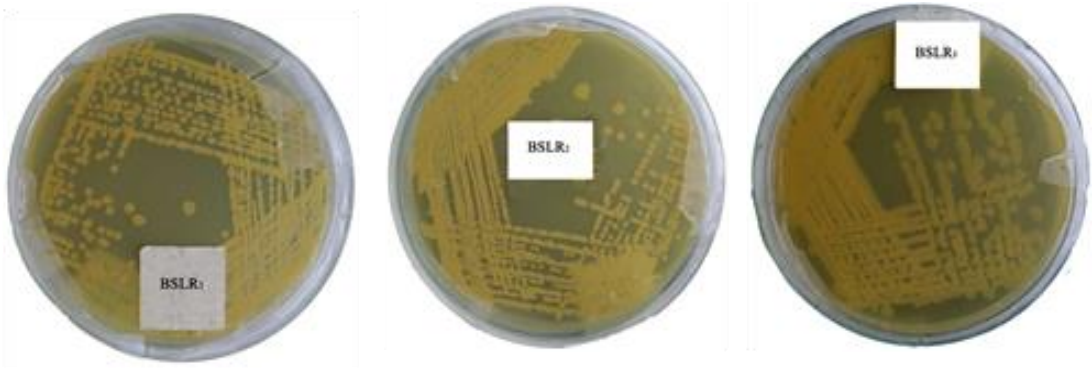


Fig 7: Culture Plates bearing isolated single colonies of isolates

Table 1: Microscopic observation of the bacterial strains

Isolates	Shape	Margin	Elevation	Size	Texture	Pigmentation	Appearance
$BSLR_1$	Round	Entire	Convex	Moderate	Smooth	Yellow	Shiny
$BSLR_2$	Spherical	Entire	Convex	Moderate	Textured	Pale yellow	Dull
$BSLR_3$	Ovoid	Entire	Convex	Moderate	Smooth	Yellow	Shiny

Elaboration: BSLR= Bacterial Sample from *Labeo rohita.*

Table 2: Gram staining

Morphological Test	$BSLR_1$	$BSLR_2$	$BSLR_3$
Gram staining Test	Positive	Positive	Positive

Elaboration: BSLR= Bacterial Sample from *Labeo rohita.*

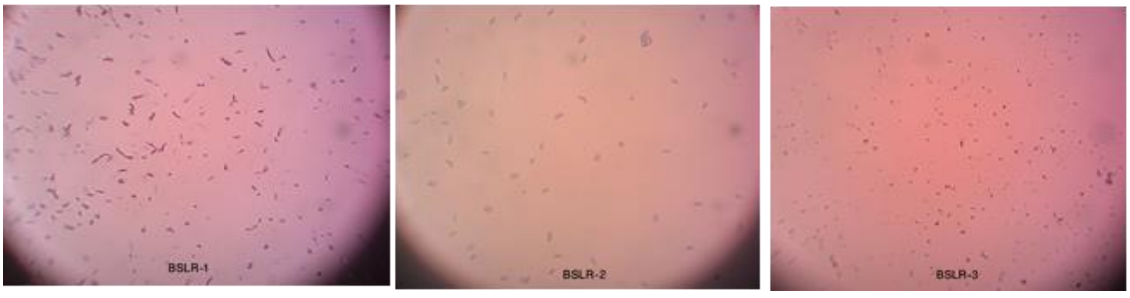


Fig 8: Gram staining ($BSLR_1$, $BSLR_2$ & $BSLR_3$)

Biochemical identification

Biochemical tests were performed to confirm the identification using oxidase, catalase, KOH, Triple Sugar Iron, Sulphur Indole Motility, Simmon Citrate, MacConkey, Kligler, Methyl Red and Urease tests. All bacterial isolates exhibited negative reactions in oxidase, potassium hydroxide, Simmon citrate, MacConkey, methyl red and urease tests as well as positive reactions in catalase test. $BSLR_3$ showed positive reaction in triple sugar iron test while $BSLR_1$ and $BSLR_2$ were found to be negative. $BSLR_2$ were positive in sulphur indole motility test but $BSLR_1$ and $BSLR_3$ showed negative. $BSLR_2$ and $BSLR_3$ exhibited positive reactions in Kligler iron test but $BSLR_1$ was negative. Biochemical characterization assumed that the isolates might be *Staphylococcus spp* (Table 3 and Fig. 9).

Table 3: Biochemical Test

Biochemical Test	Bacterial samples			Comments
	$BSLR_1$	$BSLR_2$	$BSLR_3$	
Oxidase	–	–	–	Yellow/ No Change
Catalase test	+	+	+	Bubble formation
KOH	–	–	–	Stringy Consistency
Triple Sugar Iron	–	–	+	Red, No fermentation
Sulphur Indole Motility	–	+	–	No Bacterial movement
Simmon Citrate	–	–	–	Blue/ NoChange
MacConkey	–	–	–	No change/decolorization
Kligler	–	+	+	Red to Yellow
Methyl Red	–	–	–	Yellow (No change)
Urease	–	–	–	Colorless/NoChange

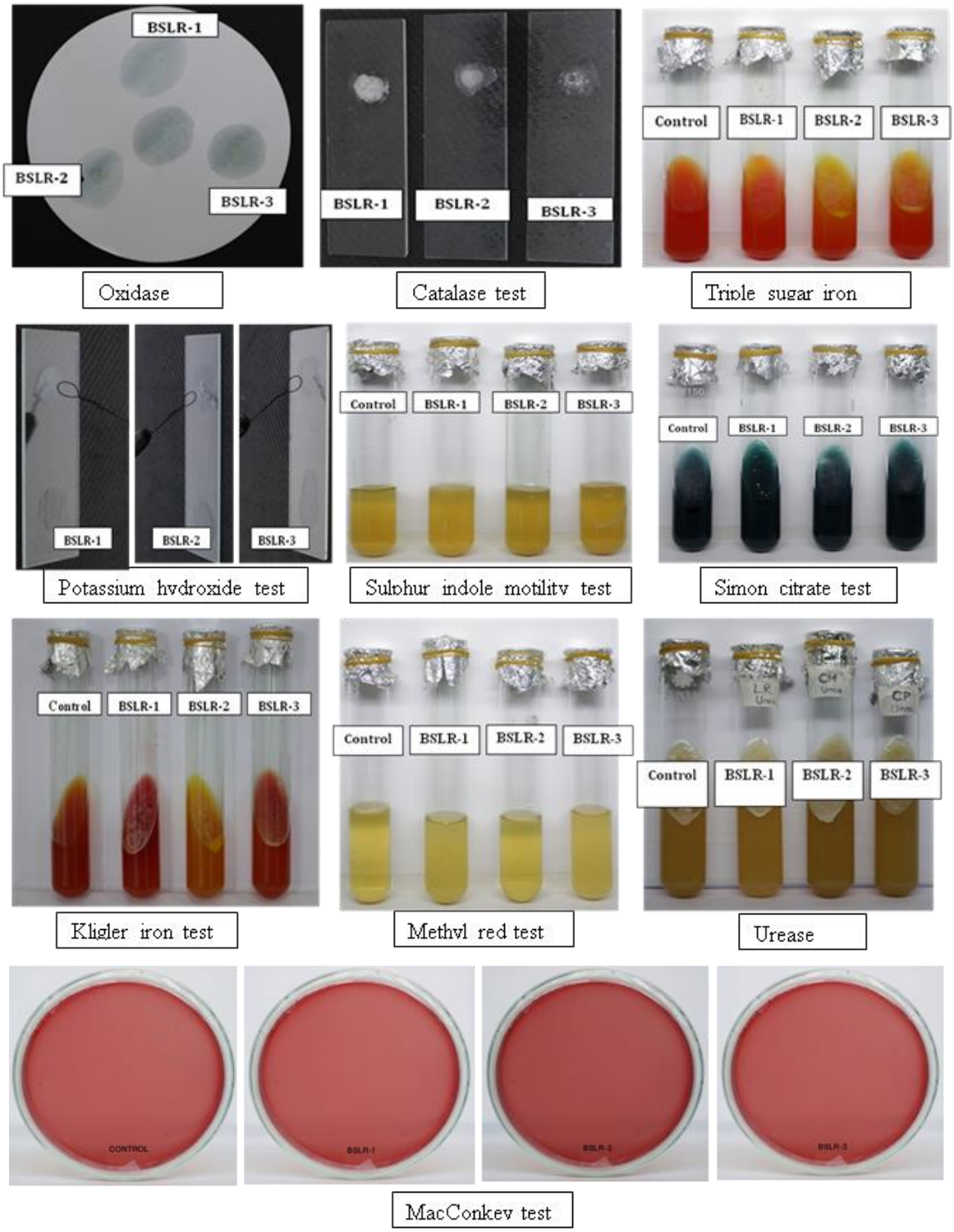


Fig 9: Biochemical identification of *Staphylococcus aureus*

Antibiotic Sensitivity Test

Antibiotic sensitivity test was performed in this study and the result was predicted based on the minimum inhibitory concentration zone. All bacterial isolates were showed sensitive to Erythromycin, Azithromycin and Gentamycin showing the zone of inhibition (mm) 21, 25 & 23, 28, 31 & 27 and 19, 21, 18 respectively whereas resistant and intermediate to Oxytetracycline (zone of inhibition 6, 10 & 12 mm) and Neomycin (zone of inhibition 10, 8 & 9 mm) respectively. $BSLR_1$ showed intermediate whereas $BSLR_2$ and $BSLR_3$ were sensitive to Doxycycline producing the zone of inhibition 13, 19 and 22 mm respectively. $BSLR_1$ and $BSLR_2$ exhibited sensitive to cefotaxime with the zone of inhibition 27 & 24 mm respectively but $BSLR_3$ (21mm) were intermediate. $BSLR_1$ exhibited intermediate with the zone of inhibition 14 mm to Sulfonamide and the rest of the two were sensitive imparting the zone of inhibition 20 & 22 mm (Table 4 and Fig. 10).

Table 4: Antibiotic Sensitivity or resistance showed by isolates against different antibiotics

Antibiotic discs	Bacterial strains			MIC zone (mm)	Remark
	$BSLR_1$ (mm)	**$BSLR_2$ (mm)**	$BSLR_3$ (mm)		
Erythromycin 15	21	25	23	21	Sensitive
Azithromycin 15	28	31	27	18	Sensitive
Cefotaxime 30	27	24	21 (I)	23	Sensitive & Intermediate
Oxytetracycline 30	6	10	12	15	Resistant
Doxycycline 30	13 (I)	19	22	16	Intermediate & Sensitive
Neomycin 30	10(I)	8 (I)	9 (I)	15	Intermediate
Gentamycin 10	19	21	18	15	Sensitive
Sulfonamide 300IU	14 (I)	20	22	17	Intermediate & Sensitive

Fig 10: Antibiotic sensitivity pattern of the isolates

Molecular identification

Three bacterial strains were identified from the isolates of $BSLR_1$, $BSLR_2$ & $BSLR_3$ shown in the Table 5 and Fig. 11-12.

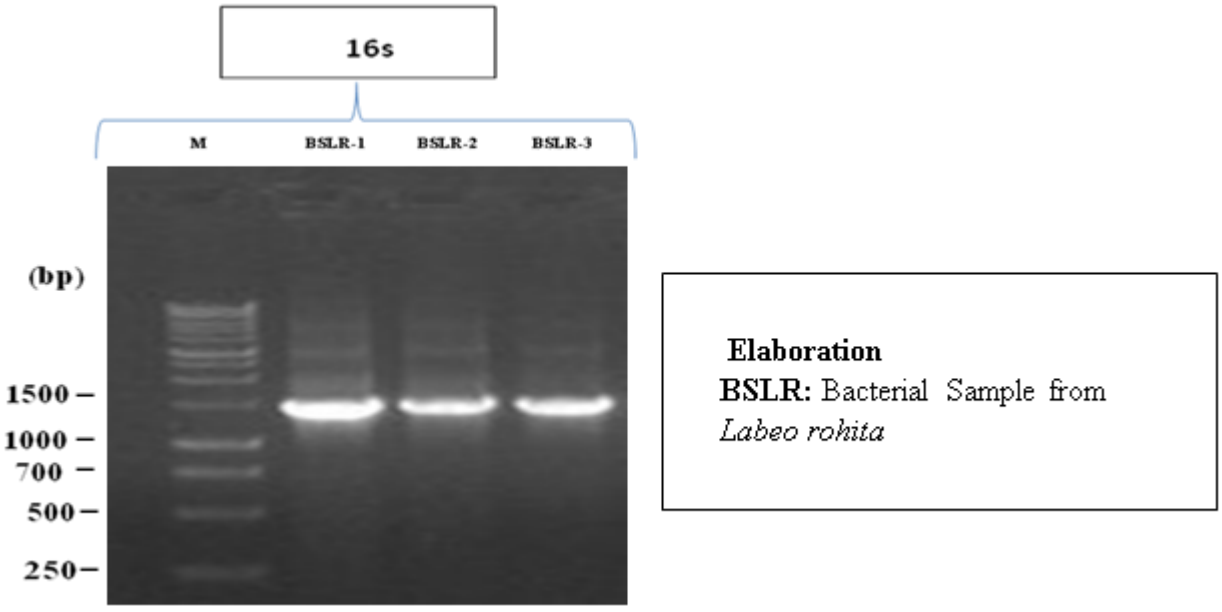


Fig 11: Purified PCR products using, primer generated brilliant single band of three isolates of the bacteria obtained from diseased fish. PCR product was run on 1% agarose gel containing Ethidium bromide. Lane 1-3: $BSLR_1$, $BSLR_2$ & $BSLR_3$, Lane 10: Tiangen M:1 kb plus DNA ladder marker.

Table 5: Isolates with 16s rDNA sequence match results found in NCBI database.

Isolates	Resembles with NCBI BLASTn Suite			
	1st Matching	Identities (%)	Sequence ID	Family
$BSLR_1$	*Staphylococcus aureus* (strain NBRC 100910)	1074/1155(93%F, 92%R)	gbNR113956.1	Staphylococcaceae
$BSLR_2$	*Staphylococcus aureus* (strain ATCC 12600)	343/364(94%F, 89%R)	gbNR115606.1	Staphylococcaceae
$BSLR_3$	*Staphylococcus aureus* (strain S33 R)	474/513(92%F, 90%R)	gbNR037007.2	Staphylococcaceae

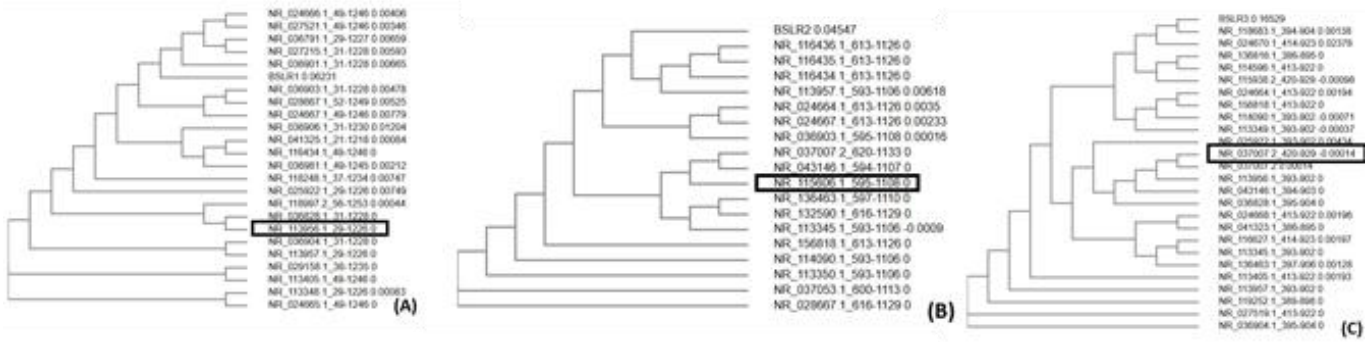


Fig 12: Unrooted phylogenetic tree for the isolates of $BSLR_1$ (A), $BSLR_2$ (B) and $BSLR_3$ (C) isolated from *Labeo rohita.* Rectangular box indicates the match.

Multiple sequence alignment among three stains of *Staphylococcus aureus*

```
BSLR1 TCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAG    174
BSLR2 TCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAG    960
BSLR3 TCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAG    960
************************************************************

BSLR1 GCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAAC    234
BSLR2 GCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAAC    1020
BSLR3 GCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAAC    1020
************************************************************

BSLR1 GGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG    294
BSLR2 GGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG    1080
BSLR3 GGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG    1080
************************************************************

BSLR1 AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG    354
BSLR2 AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG    1140
BSLR3 AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG    1140
************************************************************

BSLR1 TCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTA    414
BSLR2 TCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTA    1200
BSLR3 TCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTA    1200
************************************************************

BSLR1 GGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA-----------    463
BSLR2 GGGAAGAACAAGTGCTAGTTGAATAAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGAT    1260
BSLR3 GGGAAGAACAAGTGCTAGTTGAATAAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGAT    1260
*************************** * *.. *: ** * *:**

BSLR1 --CCAGAA-AGCCACGGCTA--A---------CTACGTG---------------------- 488
BSLR2 ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTA    1320
BSLR3 ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTA    1320
**:*:.. ***** *: * ***.***

BSLR1 ----------------------CCAGCAGCCGCGGTAATACGTAGGTGGCAAG-CGTTA-          524
BSLR2 GTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGG---CCGCAAGGCTGAAA    1377
BSLR3 GTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGG---CCGCAAGGCTGAAA    1377
 *.. *.*** ** *.**** ***** *:*

BSLR1 ---TCCGGAATTATTGGGCGTAAAGCGCGCGCA------------GGTGGTTTCTTAAG-    568
BSLR2 CTCAAAGGAATTGACGGG-----GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA    1432
BSLR3 CTCAAAGGAATTGACGGG-----GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA    1432
:..******.:  ***.** ***.** *******.:*:.*

BSLR1 ------------------------TCTGATGTGAAAGC---CCACGGCTCAACCGTGGAG    601
BSLR2 AGCAACGCGAAG-----------------------------------------------    1444
BSLR3 AGCAACGCGAAGBSLRNNNNNNNNNATNATNTGATAGTGNNTCNCG---AGATCGT----    1485
************ * *.. *: ** *

BSLR1 ------------------------------ACACCAG-------TGGCGAAGGCGACTT    708
BSLR2 ------------------------------------------------------------    1444
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BSLR3 CGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT 1724
.** ***.** ********.:*:.*
BSLR1 TCTG-----GTCTGT--AACTGACACTGAGGCGCGAA------AGCGTGGGGA----GCA 751
BSLR2 ------------------------------------------------------------ 1444
BSLR3 GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA 1784
.** ***.** ********.:*:.*
BSLR1 AACAGGATTA-------------GATACCCTGGTA-GTCCACGCCGT--AAACGATGAGT 795
BSLR2 ------------------------------------------------------------ 1444
BSLR3 GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG 1844
.** ***.** ********.:*:.*
BSLR1 ------------------------------------------------------------ 1444
BSLR2 ------------------------------------------------------------ 1444
BSLR3 CTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCTGATCCGCGATTACTA 2676
.** ***.** ********.:*:.*
BSLR1 ------------------------------------------------------------ 1444
BSLR2 ------------------------------------------------------------ 1444
BSLR3 GCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGT 2736
.** ***.** ********.:*:.*
BSLR1 ------------------------------------------------------------ 1444
BSLR2 ------------------------------------------------------------ 1444
BSLR3 GGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGTGTGT 2796
.** ***.** ********.:*:.*
BSLR1 ---------------------------------------- 1444
BSLR2 ---------------------------------------- 1444
BSLR3 AGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCC 2835
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Artificial infestation

Artificial Infestation was conducted at 100 µl, 200µl and 300µl solution of indentified bacterial strains injected below the anal and caudal fins of carp fish (Fig. 13). All isolates of $BSLR_1$, $BSLR_2$ and $BSLR_3$ infested the carp fish at 300µl bacterial suspension and did not show any infestation at 100µl concentration. At 200µl concentrations, $BSLR_1$ and $BSLR_3$ showed infestation in aquarium-2 but did not infest the fish in aquarium-1 & 3. $BSLR_2$ infested carp fish in aquarium-1 & 3 whereas no infestation was found to adhere in aquarium-2 at 200µl concentrations (Table 6).

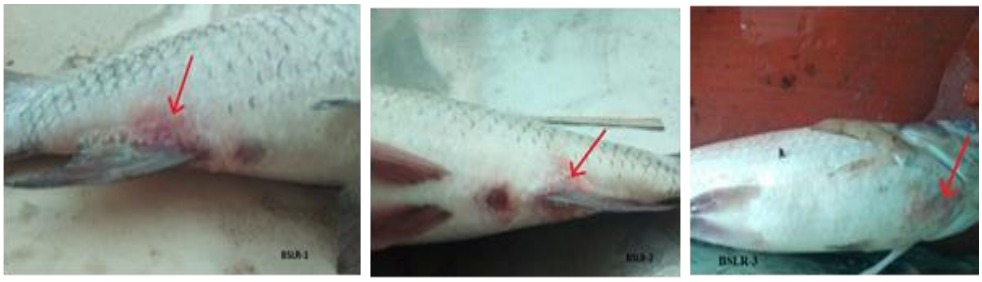

Fig 13: The carp fish was infested by identified known three bacterial isolates, $BSLR_1$, $BSLR_2$ and $BSLR_3$ of *Staphylococcus aureus*.

Table 6: Artificial infestation on carp fish by different concentrations of indentified bacterial strains.

Carp fish	Identified bacterial strains								
	$BSLR_1$			$BSLR_2$			$BSLR_3$		
	100µl	200µl	300µl	100 µl	200 µl	300 µl	100 µl	200 µl	300 µl
Carp fish, A-1	NI	NI	I	NI	I	I	NI	NI	I
Carp fish, A-2	NI	I	I	NI	NI	I	NI	I	I
Carp fish, A-3	NI	NI	I	NI	I	I	NI	NI	I

Elaborations: A= Aquarium, NI= Not Infected, I=Infected

Discussion

The present study was conducted to isolate and identify the pathogenic bacteria from diseased fish. The study showed that *Staphylococcus aureus* was the main pathogenic bacterium which was responsible for the lesions and wounds in *Labeo rohita*. The result of this study revealed that *Staphylococcus aureus* was the pathogenic bacteria found to be associated with the disease in L*abeo rohita* in Rajshahi city corporation area. The artificial infestation was conducted on carp fish by the identified bacterial strains of *Staphylococcus aureus*. *Staphylococcus aureus* is an aerobic and facultative anaerobic organism that forms fairly large yellow or white colonies on nutrient agar media. The yellow colour of the colonies was imparted by carotenoids which were produced by the organism [24]. *Staphylococcus aureus* is Gram-positive bacterium which is cocci-shaped and tends to be arranged in clusters (grape like). These organisms can grow aerobically or anaerobically at temperatures between 18^0C and 40^0C. *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus dysenteriae*, *Staphylococcus aureus* and *Staphylococcus typhi* were isolated from two edible fish of *Megalaspis cordyla* and *Priacanthus hamrur* [25]. *Staphylococcus aureus* was isolated

and identified from raw fish [26]. The pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Enterococcus faecalis, and Shigella dysenteriae* were isolated and identified from Tilapia zillii and *Oreochromis mossambicus* [27]. The presence of *Staphylococcus aureus* was attributed to the contamination of the water bodies. The pathogenic state of species of *Streptococcus* is alarming and it becomes important causative agent in the aquaculture industries [28]. *Staphylococcus* species are the most important pathogenic bacteria in fishes and some are potential pathogens as well as the high population of these bacteria indicated the degree of the spoilage in fish [29, 30].

The present study showed the similarity with Rani, MK *et al.* [31] where *Staphylococcus aureus* was isolated and identified from marine fish *Scomberomorus guttatus* and it showed positive reactions in gram staining and negative reactions in motility, MacConkey, SIM, potassium hydroxide, hydrogen peroxide, triple sugar iron and oxidase tests but negative in Simmon citrate.

In addition to this, the present study was found to be similar with Haifaa Hussein Ali [32] isolated and identified *Staphylococcus aureus* and *Staphylococcus epidermidis, Staphylococcus aprophyticus, Staphylococcus intermedius* and *Staphylococcus. hyicus* from fresh water carp fish (*Cyprinus carpio*) and Cat fish (*Silurus glanis*) where it showed the negative reactions in oxidase and other motility tests as well as positive reactions in catalase test but dissimilarity with the positive results of methyl red, mannitol, coagulase and urease tests.

Petronillah Rudo Sichewo *et al.* [33] isolated and identified the pathogenic bacteria of *Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Enterococcus faecalis* from the edible fish. *Staphylococcus aureus* was isolated and identified from *Cirrhinus mrigala, Cyprinus carpio, Catla catla, Labeo rohita, Anabas testudineus, Channa striatus, Wallago attu* and *Clarias batrachus* as well as showed the positive reactions in catalase, coagulase, thermonuclease, anaerobic utilization of glucose and mannitol tests [34]. *Staphylococcus aureus* was identified from fresh water fish where the isolates were found to be gram positive and were able to ferment mannitol, glucose, trehalose sugar and positive to coagulase, catalase and DNase tests as shown in this study [35]. The similarity was observed with the study of Nashwa A. Ezzeldeen [36] reported that *Staphylococcus aureus* isolates were identified and confirmed by PCR using two oligonucleotides primers of genes Sau 234-1501 and COAG2-COAG3 as well as the antimicrobial sensitivity revealed that *Staphylococcus aureus* isolates were sensitive to ciprofloxacin, cefotaxime, gentamycin, streptomycin, neomycin, amikacine, vancomycine, methicillin, erythromicine, aminopenicillin and tetracycline.

Gentilini *et al.* [37] observed that *Staphylococcus aureus* isolates were sensitive to gentamycine but Dendani *et al.* [38] studied that there was no resistance of *Staphylococcus aureus* isolates was found to gentamycine. Eok *et al.* [39] reported that *Staphylococcus aureus* were sensitive to vancomycine. Moreover, *Staphylococcus aureus* was found to be intermediate to neomycin in present study but *Staphylococcus aureus* isolates were sensitive to neomycin which is nearly agreed with that obtained by Malinowski *et al.* [40] It was found that *S. aureus* isolates were susceptible to erythromycin [41]. However, the present study showed similarity with *Staphylococcus aureus* isolates exhibited sensitive to erythromycin [42, 43, 44]. It was recorded that *Staphylococcus aureus* revealed sensitive to cefotaxime but one isolate of *Staphylococcus aureus* in the present study showed intermediate [45]. Similarly it was studied that the molecular and biochemical confirmation of *Staphylococcus aureus* were carried out by PCR analysis and biochemical reactions [46]. Molecular identification by PCR using different DNA targets has been used successfully for the identification of *Staphylococci* at the species level. Molecular identification of isolated *Staphylococcus aureus* strains by PCR using 2 oligonucleotides primers of genes Sau234-150 and COAG2-COAG3, which were amplified 2 band with amplified length of 1261.8 & 846.8 to 1085.6 bp for both Sau 234-1501 and COAG2-COAG3 respectively. The DNA based identification systems are targeted for species specific pathogens, allows the rapid screening of a large number of pathogens simultaneously and provides definitive confirmation of pathogens [47]. The use of universal pathway genes and universal function genes whose nucleotide sequences are more conserved in bacteria as DNA targets for PCR amplification is becoming more and more frequent [48].

The artificial infestation on carp fish was performed by the identified known bacterial strains of *Staphylococcus aureus* in current study for the confirmation of the pathogenicity, which was similar with the observation of CDC [49]. As the carp fish was infested by the identified bacterial isolates, *Staphylococcus aureus* has the pathogenicity. The artificial infestation of *Staphylococcus aureus* on carp fish was occurred as *Staphylococcus aureus* produces a wide range of virulence factors that secrete various toxins and enzymes which are responsible for the lesions during the development of the infestation. *Staphylococcus aureus* forms biofilm and penetrates as well as adheres to the host cell matrix and reaches the blood stream that can infect almost any tissue and organ [50, 51]. The virulence factors are responsible for the disease formation. The presence of these virulence factors in identified bacterial species will not only confirm the species identification but also confirm the artificial infestation and specific disease formation. When the environmental conditions are not stable, like sudden change in salinity, temperature, dissolved oxygen, pH or electrical conductivity etc. such changes become conducive for growth and proliferation of *Staphylococcus aureus* on the host organism. Poor pond management practices and higher stocking rate often resulted into outbreaks of this disease which leads to mass mortality in fish [52]. Therefore, the current study was conducted to identify *Staphylococcus aureus* isolated from *Labeo rohita*. Therefore, our study has the similarity with described above. More comprehensive works are to be solicited.

Conclusion

Observation of Clinical signs of the diseased fish, *Labeo rohita* was performed. Based on the colony morphology and pigmentation, the isolates were subjected to biochemical and molecular tests. The isolates were predicted as *Staphylococcus spp* by the biochemical characterization. Molecular characterization confirmed the species as *Staphylococcus aureus.* Three bacterial strains were identified by biochemical and molecular characterizations. Carp fish was artificially infested with identified bacterial strains of *Staphylococcus aureus*. The additional work of this study is the reisolation and reidentification of the identified known bacterial strains of *Staphylococcus aureus* from infested carp fish for further confirmation of artificial infestation.

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