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Original Research Article

From pathogen to protection: assessment of vaccination of fish

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ABSTRACT

Background: Common pathogens in the fisheries sector are primarily bacterial contaminants that are the sources of severe economic losses due to high mortality rates, with *Aeromonas, Staphylococcus, Vibrio, Pseudomonas* species are primarily very pathogenic to farm fishes in India.
Methods: Experimental fish were vaccinated with bath immersion method and injected intra-peritoneal route with subsequent experimental infection. The vaccine was evaluated and the prepared vaccine was tested for sterility.
Results: Treatment i.e. polyvalent T5, T6 and survival (after challenge) of experimental group obtained using Fisher's exact test. The resulting p value of 0.0084 ($p<0.05$) indicated statistically substantial association between treatment type and median number of survivals. Vaccine type T6 has significantly greater number of survivals as matched to controls.
Conclusions: Experimental trials of formalin inactivated vaccine via bath immersion were found to be much more effective and significant in fingerlings stage while in case of adult fish, result of intraperitoneal injection was observed as significant. As results revealed that vitamin C along with T5 i.e. T6, might have been established more immunity than others formulation doses.

Keywords: Fish bacterial pathogen, Vaccine, Protection, *Aeromonas*

INTRODUCTION

India is third major producer of fish and the second major producer of fresh water fish in global market. The fisheries industry of India has been playing a dynamic role in the Indian economic growth by advantage of its prospective involvement to employment creation, income progress, food and nutritional security concern and foreign earnings. Freshwater aquaculture production provides nearly 55 percent of the total fish income in India. Some of the important species cultured in India are 18 species of carps, which are cultured in the region major carps like *Labeo rohita, Catla catla, Cirrhinus mrigala, Cyprinus* carpio and *Clarius sp.* Besides, these ornamental fish culture and seaweed farming, are gradually achieving position in the last few years.

With the growing commercialization of aquaculture industry, disease is a major concern which reasons illness and death, reported from hatcheries and nurseries in the fish industry. Several bacterial, parasitic and fungal diseases have been reported in farming water systems working culture-based fisheries and can cause infection either as main pathogens or as secondary cunning attackers. Poor water feature, organic contaminants, temperature fluctuations and extremes, wild fish reservoir, stress, overcrowding, poor nutritional position and trauma could easily predispose early developmental stages to bacterial infections. As the aquaculture industry increases, apparatuses to observe the health position of commercial/farmed fish expending standardized and inexpensive systems will be needed. The pathogenic factors (infectious and non-infectious) cause to reduce in production in fresh water fish farms.[1] To solve these problematic issues, many approaches such as disease resistant fish, an elevating diet, vaccination, antibiotic and immunostimulants must be developed. There is, therefore, a need to continue to strengthen regional and international

cooperation in aquatic animal health. Infected fish were swimming close to the water surface, lethargic, showed loss of balance and hyperventilated. Some developed ascites and petechial hemorrhages, especially around the anal zone.[2] It was found that *streptococcus* infection detected in high prevalence among cultured fresh water fishes especially during summer seasons. *Aeromonas sp.*, is considered a truly opportunistic pathogen, because it is relatively common in the aquaculture environment (hence, the term "environmental bacteria") and typically does not cause disease in healthy, well-maintained fish populations. Skin acts as a mirror for health condition of the fish in aquaculture industry. Since some pathogens attack the skin not only due to surface contamination from aquaria but also due to invasion by pathogenic microorganisms. The skin ulcers may occur at any site on the fish and often a bright red rim of tissue surrounds them.

From last decade or more, great advances have taken place in understanding the vaccination of humans and other animals induces specific immunity to assist in the elimination of microbes, neutralization of microbial toxins and prevention of further microbial invasion and identified as an active immunization that results in increase in the concentration of naturally acquired antibodies.[3,4] The purpose of vaccination is to induce protective immunity in an animal. The immunity is specific and should be long lasting. Vaccination is prophylactic and is much more effective against infectious diseases compared to other type's treatments like antibiotic treatment. In aquaculture, the vaccine should be effective and applicable compared to the most economically important diseases. The commercially available vaccines used against fish infectious diseases contain formalin inactivated bacterial cells. The vaccines contain adjuvant, which increase the efficacy towards health of fish. The market available salmon vaccines in Norwegian aquaculture are oil-based adjuvant vaccines and are most often multivalent vaccines, containing up to seven different components. The current advances in technology seem to offer a promising future for antibacterial vaccines in fish species. However, it is more difficult to develop effective and cheap anti-viral vaccines and anti-parasitic vaccines.[5]

The vaccine was evaluated and the prepared vaccine was tested for sterility. Experimental fish were vaccinated with bath immersion method and injected intra-peritoneal route with subsequent experimental infection (challenge dose).

METHODS

Field survey of aqua –reservoirs

There are no major riverine fisheries in Nagpur district. Of the rivers of Nagpur only Waingangā, Wardha, Kanhan and Pench are important from the fishery point of view. The fisheries in the district are located along the tanks and lakes. Many analytical methods, which are combination of physico-chemical, biochemical, and microbial procedures, have been adopted for surveyed water samples from various reservoirs. The methods were intended to obtain qualitative data of level of contamination of the reservoirs and ultimately their hazardous effects on the existence of aqua culture, especially fish fauna.

Collection of different samples

The samples were experimented from the most significant lakes and tanks in the district from fisheries perspective, where fish culture is being undertaken either in public or in reserved sector.

Physico-chemical analysis of water samples from various aqua-reservoirs

The abovesaid water samples from surveyed lakes and reservoirs were collected, as these are exposed to constant variations from normal causes such as seasonal stratification, rainfall, run off and wind. In the present investigation water sampling had to be dependent upon the local condition and for the preservation of their integrity. They were subjected for varied physico-chemical parameters in the laboratory. The analyses were carried out immediately to avoid any significant changes, particularly for parameters like temperature, pH, dissolved oxygen, biochemical oxygen demand, total hardness, total alkalinity and chemical oxygen demand.

Samples collected as mentioned above, for further microbial examination. The microbial examination of water samples was carried out promptly after collection to avoid unpredictable changes.

Determination of fifty percent lethal dose ($LD_{50\text{-}96}$ hours)

The fish were preserved in a continuous water system with aeration and observed for 96 hrs. $LD_{50\text{-}96}$ hours values were calculated according to the method.[6]

Monovalent and polyvalent formalin inactivated vaccine

For preparation of monovalent formalin inactivated vaccine, as T5, T6 while Tc was control. Isolated culture was inoculated into brain heart infusion broth and incubated at 30 ºC for 24 hours under continuous agitation. Broth of 5×10^6 CFU per ml culture and formalin (0.5% V/V) was added to the broth culture at a final concentration and left for 48 hours. at room temperature with continuous agitation. Formalin inactivated bacterial cultures were centrifuged at 4,000 g for 30 min, and supernatant were discarded and re-suspended in phosphate buffer saline.

Bath immersion

For the treatment T5, T6 vaccine were experimented 1:10 part dissolved in water for bath immersion. The vaccination doses were used and fish were kept in bath for 20 mins. Each glass aquarium organizing both vaccinated and control fish.

Table 1: Formalin inactivated vaccination and control groups of Rohu fingerlings using bath immersion route.

S. no.	Type of vaccine	No. of fingerlings	No. of controls
1	T5	10	10
2	T6	10	10
5	Tc	10	10

Challenge study

For challenge study, 24-hour brain heart infusion broth culture was used. The culture was diluted 1:10 in PBS. Twenty eighth days after immunization each group were challenged with culture.

Deaths were monitored and any quantifiable symbols in survivors were identified.

RESULTS

The present study was carried out on different specimens which were randomly collected at different seasons during field survey. All data were collected and recorded very precisely during field surveys which provide detailed information about the Nagpur region, fish health, maintenance and water quality of lakes and tanks.

Physico-chemical analysis of water samples from various aqua-reservoirs

In the present study, variation of several water samples from various aqua-reservoirs were collected and brought in to laboratory for studying physicochemical parameters like pH, temp, dissolved oxygen (DO), biochemical oxygen demand (BOD), total hardness (TH), total alkalinity (TA), chemical oxygen demand (COD) were analysed and characterized accordingly. Nevertheless, their values as well as their behaviour depend significantly on the type of collected water samples. Studies of water quality in various samples revealed that anthropogenic activities have an important negative impact on water quality in the downstream sections of the major water reservoirs in Nagpur. This is a result of cumulative effects from upstream expansion of other activities but also from inadequate wastewater treatment facilities. Different results have been obtained for water samples collected from reservoirs. Most of the chemical parameters are within accepted ranges. The analysis of different water parameters of water samples from various aqua-reservoirs were recorded: pH, dissolved oxygen, biochemical oxygen demand, total hardness, total alkalinity, chemical oxygen demand, and temperature.

Microbial analysis of different water samples

All samples were transported to laboratory in suitable autoclaved container and analysed on the same day within 24 hours of collection. For water samples, membrane filter technique was used. Water samples were filtered with measured volume i.e. 100 ml through the membrane. The membrane filter method is applied for water samples during study except those having high turbidity which may block the membrane. Standard plate count (SPC) method was also done for microbial analyses of different water samples drawn from various aqua reservoirs (Table 2).

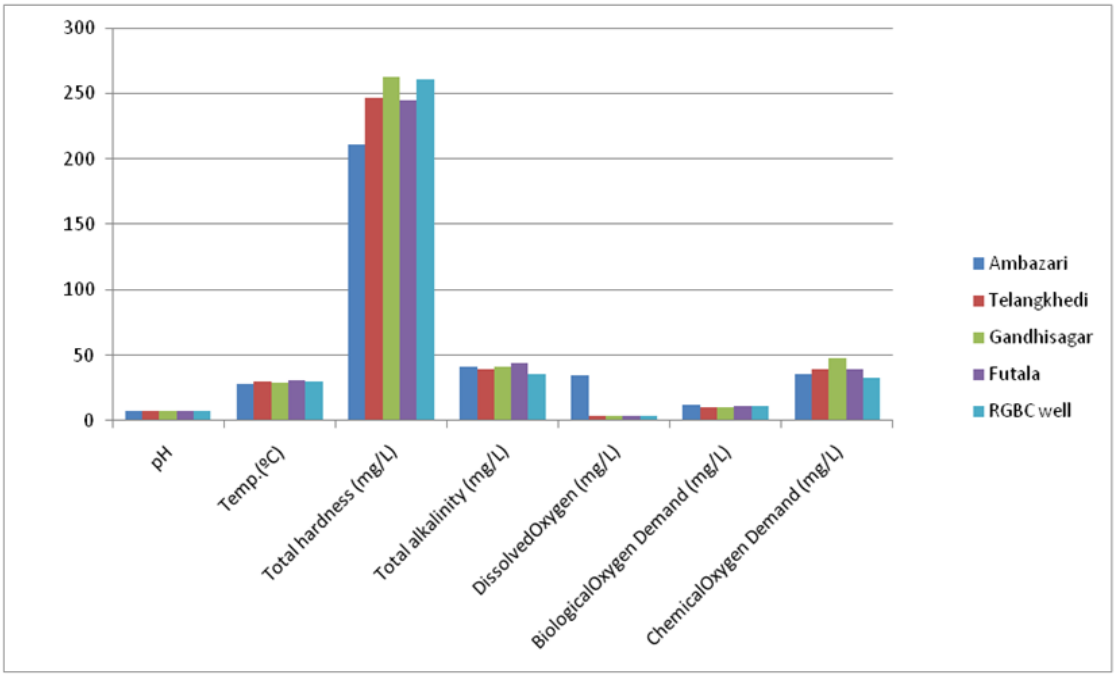

Figure 1: Analysis of different physico-chemical parameters of water samples (average of three replications).

Table 2: Microbial analysis by standard plate count of different water samples drawn from various aqua reservoirs (average of three replications).

S. no.	Location	Bacteria CFU/ml	Fungal (propagals/ml)	Total MPN/100 ml	
1	Ambazari		23×10^5	12×10^2	248
2	Telangkhedi		29×10^5	9×10^2	300
3	Gandhisagar		28×10^5	14×10^2	262
4	Futala		30×10^5	16×10^2	290
5	R.G.B.C well		19×10^5	7×10^2	213

Symptoms observed during the survey

The clinical signs of infested fish revealed pathogenic abnormalities on the external body surface. The autopsy of the fish showed that the internal organs were appeared anaemic with enlargement and congestion, haemorrhage ulceration of intestine, stomach mucous membrane, disintegration of liver and other organs commonly affected with disease include the gills, kidneys, spleen, and pancreas. Bacterial flora was isolated simultaneously from the surface lesion of affected fishes as well as, from, their muscles, gut, liver, gills, heart, kidney and gonads. The symptoms of disease like red spots or red wounds found exudation blood and fluid from skin, swimming abnormalities, pale gills, bloated appearance, and skin ulcerations. The skin ulcers may occur at any site on the fish; these ulcers/wounds are found all over the body of the fish especially on the stomach and tail region and often are surrounded by a bright rim of red tissue.

Isolation and study of bacterial strains and their growth conditions

For isolation and study of bacterial strains and their growth conditions, all samples from infected part were collected in Stuart transport medium for probable micro flora from tanks and lakes to the laboratory and were inoculated on nutrient agar and as well as selective media plate.

Figure 2: Samples collected in Stuart transport medium (Himedia) for preservation and transportation of organisms from various fish and lakes to the laboratory.

These bacterial colonies were subjected to characterize phenotypic and culture characterization. For thorough identification of isolates, initial data of bacterial isolation were studied and recorded precisely.

Isolation and identification of fish pathogens from different samples

During the screening of samples for bacterial isolates, the number of bacterial colony forming units (CFU) was varied.

On the basis of preliminary data collected, the screening and investigations of bacterial isolates were done to assess and identify the types of bacterial pathogens on the basis of Gram staining, motility and different cultural characteristics and thus revealed the occurrence of *Escherichia coli, Aeromonas spp., Pseudomonas spp., Staphylococcus spp., Streptococcus spp., Proteus spp.., Klebsiella spp., Salmonella spp., Vibrio spp., Bacillus cereus,* and some fungal colonies as found on selective media plate of respective organisms .

Following different media were used for screening and isolation of different organism.

Nutrient agar

Off white coloured colonies of *Staphylococcus aureus*.

Nutrient agar

On nutrient agar surface *Proteus spp*., colonies were translucent and white to buff in color, swarming growth and non-pigmented.

Bacillus isolation agar

White coloured colonies of *Bacillus spp.* were found.

Cetrimide agar

Slight green coloured, pigmented colonies of typical *Pseudomonas spp.*

Brain heart infusion agar

Typical yellow to off- white coloured, circular colonies of *Streptococcus spp.* were found and further used for *Streptococcus spp.* isolation media.

Mannitol salt agar

Typical deep yellow coloured colonies for further identification and isolation of *Staphylococcus aureus*.

Rimpler-Shotts media

All colonies which found to be yellow/yellow greenish, circular were isolated as presumptive strains of *Aeromonas spp*.

Isolation of Aeromonas spp.

In this study, all selected bacterial strains were discarded except which were positive Aeromonas spp. isolates, which were then subjected to Gram staining and motility, they were observed as Gram negative pink rod and all strains were very active and appeared motile under the microscope (Figure 3).

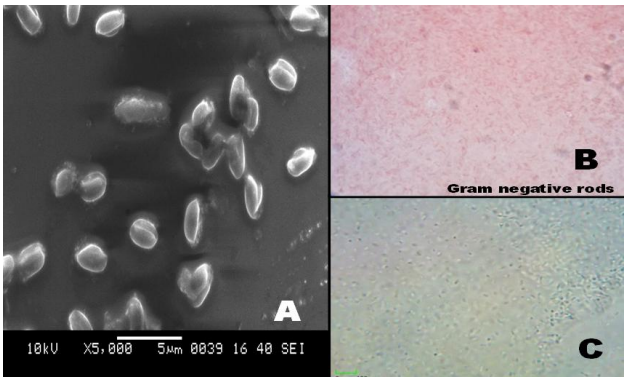

Figure 3: Figure showing (A) scanning electron microscope; (B) Gram staining of *Aeromonas spp.*, and (C) motility by hanging drop method.

Presumptive isolates were further tested for biochemical, cultural and antibiotic sensitivity. Further, all isolates were tested for various biochemical tests using *Enterobacteriaceae* identification kit for biochemical identification of presumptive Aeromonas spp. The use of *Enterobacteriaceae* identification kit for varied biochemical tests was one of the updated techniques for screening of isolates. On the other hand, drawback of using this kit is that only acid production was observed in sugar fermentation media. So, whichever sugar showed acid production, they were tested for acid and gas production for confirmatory results. Catalase test was also performed.

In the present investigation, morphological and biochemical tests for identification and isolation of *Aeromonas spp.* isolates were evaluated. Only few characteristics exhibited uniform results.

As mentioned in Table 3, the biochemical characteristics were similar with the reference strain obtained from MTCC, except for some differences in H_2S, indole, sorbitol and rhamnose production; all other showed positive results. They were Gram negative, motile, oxidase positive and catalase positive, fermentation of D-glucose, production of nitrate reduction.

There were 18 isolates of *Aeromonas spp.* used in this studies which were subjected to different biochemical identification test.

Table 3: Biochemical properties of presumptive *Aeromonas spp*.

S. no.	Biochemical properties	*A. hydropila* MTCC 1739	No. of isolates of *Aeromonas* spp. showing peculiar character (n=18)
1	Gram staining	-	- (18)
2	Motility	+	+ (18)
3	Adonitol	-	- (16)
4	Arabinose	-	- (10)
5	Catalase	+	- (14)
6	Cellobiose	-	- (15)
7	Citrate utilization	+	+ (17)
8	Esculin hydrolysis	+	+ (16)
9	Glucose	+	+ (18)
10	H_2S production	-	+ (06)
11	Indole	+	+ (12)
12	Lactose	-	- (16)
13	Lysine utilization	+	+ (16)
14	Malonate	-	- (14)
15	Mellibiose	-	- (17)
16	Methyl red	-	- (13)
17	Nitrate reduction	+	+ (18)
18	ONPG	+	+ (17)
19	Ornithine utilization	-	- (16)
20	Oxidase disc	+	+ (18)
21	Phenylalanine deamination	+	+ (14)
22	Raffinose	-	- (13)
23	Rhamnose	-	- (17)
24	Saccharose	+	+ (17)
25	Trehalose	+	+ (15)
26	Urease detection	-	- (17)
27	Vogus Proskauer's	+	+ (15)
28	Xylose	-	- (17)

Note: ONPG- o-nitrophenyl-β-D-galactopyranoside

Further, the selective media plates with yellowish-green or yellow colored colonies were exposed to iodine vapors which showed clear zone around the colonies of *Aeromonas spp*. and the production of H_2S showing black colors stab was also found to be a typical character of identification of *A. hydrophila* subsp. hydrophila.

For identification and confirmation of the genus, following differential media were used.

Sheep blood agar

Only few of the isolates were showed pale-yellow coloured colonies with clear zone.

MacConkey agar

Pale coloured, opaque colonies were found and confirmed as non-lactose fermented bacteria.

Xylose lysine deoxycholate agar

On xylose lysine deoxycholate agar surface red coloured colonies were found.

Aeromonas selective agar base (Ampicillin supplement)

On *Aeromonas* selective agar surface, yellowish-green, opaque colonies with dark centers colonies which confirmed that the selected bacterial strain belongs to genus *Aeromonas.*

Different selective media were very useful for identification and isolation of organisms along with all this, biochemical properties and antibiotic sensitivity were also done to confirm the presumptive isolates are of *Aeromonas spp*.

The following figure 4 represents the antibiotic sensitivity test for randomly selected strains of presumptive *Aeromonas spp.* isolates. The results are depicted by taking average of three replicates and zone of clearance of antibiotic sensitivity against isolates were measured in diameter (mm). The results confirmd that *Aeromonas spp.* are poorly susceptible to Norfloxacin (15 mm) and to a lesser extent tetracycline (17 mm), Co-Trimoxazole (21 mm) active similar to Nalidixic acid (21 mm), Ampicillin amd penicillin has no zone while Cefuroxime (8 mm) and Pipemidic acid (6mm) shows insignificant zone.

The following graph represents the antibiotic sensitivity test selected strains of presumptive *Aeromonas spp.* isolated in this study (Figure 4).

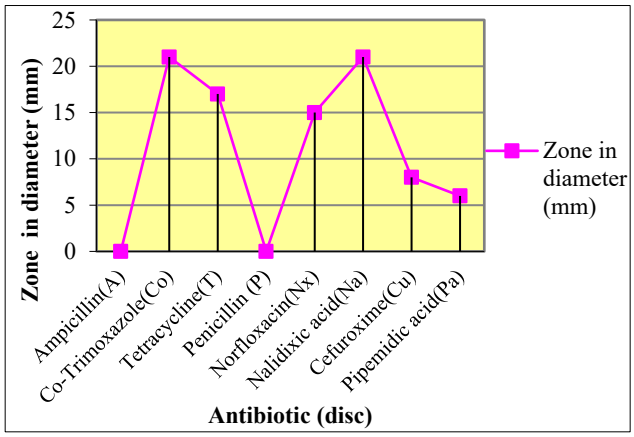

Figure 4: Depiction of antibiotic sensitivity test.

Fifty percent median lethal dose (LD $_{50\text{-}96}$ hours)

The $LD_{50\text{-}96}$ hours was determined for the ten fingerlings of Rohu weighing about 30-40 g. The result obtained in this experiment, the $LD_{50\text{-}96}$ hours doses for each strain are given in Table 4.

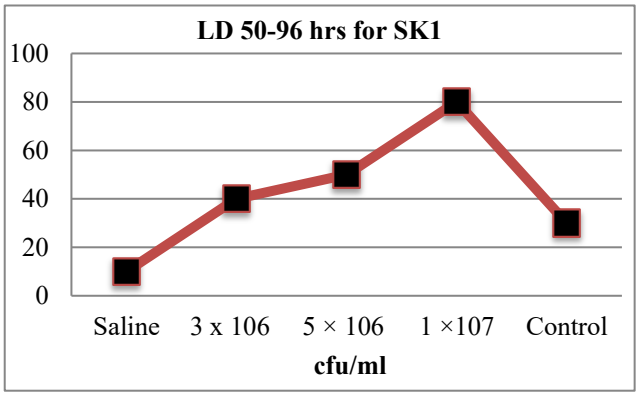

Figure 5: Representation of LD 50-96 hours for SK1 (isolated culture).

Adult fish were experimentally infected by sub-lethal $LD_{50\text{-}96}$ hours dose of *Aeromonas spp.* SK1 and results were signifying with hemorrhagic septicemia and mortality in fish.

There were 18 isolates used in these studies, which were identified on the basis of different morphological, biochemical and cultural tests identification.

All formalin inactivated vaccines were prepared and tested for their sterility by streaking it onto brain heart infusion agar, which showed no growth after 24 hours. After sterility test all vaccines were kept at 4 ºC till further use.

Figure 6: Formalin – inactivated vaccine.

Route of vaccine administration - bath immersion

Vaccination trials by bath immersion route have been Fingerling groups of rohu were vaccinated by intraperitoneal_route with different formalin inactivated vaccine either as monovalent or polyvalent. These trials were found to be significant in the survival rate for

different treatment modalities against control in 36th day of observation time.

Route of vaccine administration - intraperitoneal route

The present result evaluates the effects of polyvalent vaccination containing T5 i.e. polyvalent (*Aeromonas* spp. + *Streptococcus* spp) and T6 (T5+Ascorbic acid). The experimental and control fish were sacrificed at the end of trials and histopathological, scanning electron microscopic hematological studies of tissues were done after the experiment.

After the experiment, healthy and live fish were demonstrated considerable survival of T6 vaccine group which were showed no disintegration of any of the organ in anesthetized dissected fish. Experimental vaccine groups i.e. T5 and T6 have been survived and showed significant results. Experimental intraperitoneal administration of formalin–inactivated group i.e. T5 and T6 were survived while in case of control group all fish were showing clinical symptoms on an application of challenge/virulent dose, most of them found dead at the end of experiment. Interpretation of experimental outcome with formalin inactivated vaccine by intraperitoneal administration of vaccine groups and resulting in p-value significance according to Fisher's exact test. Overall association between the types of treatment i.e. polyvalent T5, T6 and survival (after challenge) of trial group obtained using Fisher's exact test. The resulting p value of 0.0084 ($p<0.05$) indicated statistically significant association between treatment type and median number of survivals. Vaccine type T6 has significantly higher number of survivals as compared to controls (Table 4).

Table 4: Observations of intraperitoneal administration of vaccine groups and p value significance calculated according to Fisher's exact test.[16]

S. no	Type of vaccine	Group	No. of fingerlings	No. of survival group (median)	No. of mortality (median)	P value
1	T5	Polyvalent (*Aeromonas sp.+ Streptococcus spp)*	10	7	3	0.0084 ($p<0.05$)
2	T6	T5 +Ascorbic acid	10	9	1	
3	TC	Control	10	2	8	

DISCUSSION

In the present investigation, the studies with respect to the origin of *Aeromonas spp*. The occurrence of *Aeromonas spp*. is poorly documented in Vidharbha region, only few researchers were reported about *Aeromonas* spp. infection and other bacterial diseases in aqua culture polluted water.[7] In present studies, most of the isolates of *Aeromonas* spp. were found to be susceptible to Nalidixic acid, and Co-trimoxazole while tetracycline is intermediately susceptible whereas Ampicillin and Penicillin was showed resistance.

As reported in their findings that all 65 *A. hydrophila* strains in their study isolated from either water sample or healthy and diseased fish, biochemical characteristics were variable between the isolates.[8] The biochemical results obtained with *Enterobacteriaceae* identification kit in this study were in accord with the results and conventional biochemical tests.[9,10]

In this study, finding of different selective media were very useful for identification and isolation of organisms. Further, biochemical properties and antibiotic sensitivity were also done to confirm the presumptive isolates are of *Aeromonas spp*.

As reported differences in the biochemistry between the culture isolates of *A. hydrophila* they screened, but they found these variances to be lacking to separate the isolates into virulent and avirulent groups.[11]

In present research studies, $LD_{50\text{-}96\ hours}$ value is found to be 5×10^6 CFU/ml for strain SK1.

In the present investigation, experimental trials using immunostimulants as vitamin C in combination with formalin inactivated monovalent and polyvalent vaccine. Tc having vitamin C certainly enhances both the specific and non-specific immunity of the catfish, *M. gulio*. The observed RPS values of 82-100% in common carp immunized with an *A. hydrophila* biofilm (heat inactivated) vaccine, while an RPS value of 76-81% was seen in fish immunized with heat inactivated free-cell suspension of *A. hydrophila*.[12]

It is found that injection is a more reliable method of vaccination compared with the oral or topical application of vaccines against *Aeromonas* infections. Indian carps and tilapia immunized either intramuscularly or intraperitoneally with *Aeromonas* vaccine, showed protection against challenge and the agglutinating antibody titre increased in the serum of immunized fish.[13,14] Catfish immunized intraperitoneally by injection with the acid extract of the S-layer protein of *Aeromonas hydrophila* were protected from the homologous, virulent strain.[15] In the present study, intraperitoneal administration of formalin–inactivated vaccines groups in adult fish, the histopathological study revealed vaccinated and control fish were observed after challenge dose, structural changes were seen more evident in specimens of control fish; any significant changes in tissues of fish.

CONCLUSION

Overall in the present investigation, experimental trials of formalin inactivated vaccine via bath immersion were found to be much more effective and significant in fingerlings stage as per relative percent survival (RPS). While in case of adult fish, result of intraperitoneal injection was observed. As results revealed that vitamin C along with T5 i.e. T6, might have been established more immunity than others formulation doses. But, there is also possibility that experimental adult fish under study might have previously developed protection, as it is showing to such bacteria previously or sometimes stress aspect may modify the result. Hence, the present investigation strongly proposes a new dimension for such studies for future prospective in aquaculture research.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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