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Prem Timalisina

National Fishery Research
Centre, Godawari, Lalitpur,
Nepal

Bhuwan Gurung

National Fishery Research
Centre, Godawari, Lalitpur,
Nepal

Rojina Thapa

National Fishery Research
Centre, Godawari, Lalitpur,
Nepal

Prakash Kunwor

National Fishery Research
Centre, Godawari, Lalitpur,
Nepal

Asha Rayamajhi

National Fishery Research
Centre, Godawari, Lalitpur,
Nepal

Neeta Pradhan

National Fishery Research
Centre, Godawari, Lalitpur,
Nepal

Mahendra Bhandari

Fisheries Research Station,
Trishuli, Nepal

Suraj Singh

Rainbow Trout Fishery Research
Station, Dhunche, Nepal

Anup Sharma

National Fishery Research
Centre, Godawari, Lalitpur,
Nepal

Corresponding Author:

Prem Timalisina

National Fishery Research
Centre, Godawari, Lalitpur,
Nepal

Tail and fin rot disease and antibiotics resistance pattern in major rainbow trout farms of Nepal

Prem Timalisina, Bhuwan Gurung, Rojina Thapa, Prakash Kunwor, Asha Rayamajhi, Neeta Pradhan, Mahendra Bhandari, Suraj Singh and Anup Sharma

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Abstract

Tail and fin rot is one of the most economically important disease throughout the world. Occurrence of tail and fin rot and emergence of antibiotic resistance in trout farming has mandated extensive survey, characterization of dominant causative agents followed by screening of effective antibiotics. In present study, predominant fin and tail rot causing bacterial pathogens were isolated from samples collected from fifteen rainbow trout farms of major trout pocket areas i.e. Rasuwa, Nuwakot, Dhadhing, Sindhupalchowk, Lalitpur and Kaski districts of Nepal. All the isolates were further tested for pathogenicity, which showed virulence in fancy carp (*Cyprinus carpio haematopterus*). *Pseudomonas* spp and *vibrio* spp showed more virulence. Antibiotic susceptibility test revealed that all the commonly used antibiotics (Amoxicillin, Azithromycin, Neomycin, Oxytetracycline and Streptomycin) were completely resistant. Among presently used antibiotics, Cephalexin was resistance in all the farms while Doxycycline was found completely resistant in two farms and Moxifloxacin was effective in general. Positive correlation between disease occurrence and water parameters were observed among various trout farms. Lack of knowledge regarding use of antibiotics, development of multidrug resistance bacteria and downstream effect of antibiotics used in trout farms was observed among farm's owner and farmers, implicating a need to develop an effective protocol in fresh water aquaculture in Nepal.

Keywords: Rainbow trout, tail and fin rot, pathogenicity, antibiotic, resistance

Introduction

Water availability and current trend of commercial trout farming trends suggest that Nepal could be one of the lead Trans-Himalayan countries for mountainous cold water aquaculture fish rainbow trout, especially where other agriculture activities are not feasible (Sharma 2008; Gurung TB, Wagle SK, Nepal AP, and Lamsal GP, 2017) [35, 26]. There are 120 farms producing more than 551 metric tons of rainbow trout in Nepal (NFRC, 2021) [27]. These reasons have attributed to welfare of farmed fish with increased attention in public perception with hind sight of production efficiency, quality and quantity (Broom, 1998; Southgate and Wall, 2001; FSBI, 2002) [6, 37, 14]. Tail and fin rot is one of the major trout bacterial diseases (Larmoyeux and Piper 1971; Faruk, Alam, Sarker and Kabir, 2004, Rayamajhi 1998) [22, 12, 30] and fin Damage in rainbow trout farms is regarded as operational and welfare indicator of trout farms (Ellis, Oidtmann, St-Hilaire, Turnbull, North B, MacIntyre *et al.* 2008, Ellis, Hoyle, Oidtmann, Turnbull, Jacklin, and Knowles, 2009; RSPCA 2014) [10, 11, 34]. Higher stocking density with decreased food utilization efficiency and environmental contaminants has been reported to be directly correlated with increase in fin and tail rot (Kindschi, 1987; Jorgensen, Christiansen and Jobling, 1993, Ellis, North, Scott, Bromage, Porter, and Gadd, 2002, Riley, Tataru and Scheurer, 2005) [21, 19, 9, 33]. Occurrence of tail and fin rot has also been reported at low stocking density when fish were intermittently fed (Winfree, Kindschi and Shaw, 1998) [31]. Fish stocking density and under fed trout tend to have impaired immune function (Pickering, Pottinger, Sumpter, Carragher, Le Bail, 1991; Adams, Huntingford, Turnbull, and Beattie, 1998; Dibattista, Levesque, Moon, and Gilmour, 2006) [28, 2, 8]. Previous small scale studies in Nepal have reported tail and fin rot as common chronic disease found in poorly kept trout and often occurs simultaneously with other diseases. (Nepal, Basnet, Lamsal, Joshi and Mulmi, 2002; Rayamajhi and Dhital, 2008; Rayamajhi and Prasad 2010, Jha and Bhujel 2012) [26, 31, 32, 18].

To control the spread of tail and fin rot disease various antibiotics are used (Hernández Serrano, 2005; Miranda, Godoy and Lee, 2018; Rahman, Ferdowsy, Kashem and Foyosal, 2010) [16, 24, 29]. Moreover, the resistance pattern of bacterial pathogens reflects the extensive use of antibiotics (Smith, Hiney and Samuelson, 1994; Bruun, Schmidt, Madsen, and Dalsgaard, 2000) [36, 5]. Over and misuse of antibiotics, especially when these compounds are routinely used, even in the absence of disease has resulted in antibiotic resistance (Cabello, 2006) [7]. In context of Nepal, on farm examination of the samples from the fish health camp carried out in Trishuli and Rasuwa showed that about 48% trout fry, 26% fingerlings and 13% broods samples were infected with tail and fin rot disease despite use of the antibiotics (FRD 2018) [13]. In addition, available cross sectional reports also supported similar results on food animals pointing the potential overuse or misuse of antibiotics for therapeutic or sub-therapeutic purposes (Acharya, 2011, Khatiwada, 2012) [1, 20]. An increase in the number of trout farms in the country, the persistent presence of tail and fin rot disease, and improper use of antibiotics have mandated an extensive survey, characterization of dominant causative agents followed by the screening of effective antibiotics as well as its good management practice. Therefore, the aim of the presence study is to isolate and to screen the disease causing pathogen, its pathogenicity and antibiotics profiling of tail and fin rot disease of rainbow in Nepal.

Materials and Methods

Farm Survey and Fin Rot Sample Collection

Semi-structured questionnaire and focal group discussion (FGD), was conducted in 15 farms of trout pocket area during early winter season, December-January 15, 2020 (Rasuwa-3, Nuwakot-3, Dhading-1, Sindhupalchowk-2, Lalitpur-2 and Kaski-4). Each fish farm was assigned with unique name code. From each farm, 5 fin-tail rot disease fish & asymptomatic fish as control were collected for microbiological analysis. Blood Sample was collected in vial containing sterile Brain Heart Infusion Broth whereas; kidney, liver & fin swabs were collected in sterile swab collector (Himedia).

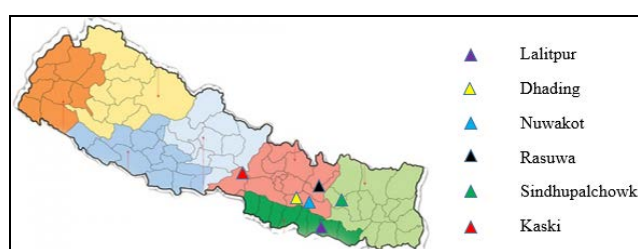


Fig 1: Survey and Sample Collection site

Water Parameter Analysis

During the Survey, instant water quality parameters (AP500) Dissolved Oxygen, pH, Temperature were recorded from each farm and water 50 ml sample was collected from each farm for Total Ammonical Nitrogen (TAN), Nitrate (NO₃), Nitrite (NO₂), Total Alkalinity (TAL) and analyzed at water quality lab of Fisheries Research Division (FRD) Godavari using eXact micro 20 strips kit. Care was taken to record water quality parameters and water sampling at same time period 9:00 am between farms.

Isolation & Screening of Pathogenic Bacteria

Isolation, identification of pathogen was conducted disease lab of National Fishery Research Centre, Godavari, Lalitpur. Blood, kidney liver and fin swab samples were enriched on Brain Heart Infusion Broth (BHI) broth at 37 °C for 24 hrs. For the selective isolation of suspected pathogen, enriched sample were streaked on Bile Salt Brilliant Green Starch Agar at 30 °C for 24 hrs, Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) at 35 °C for 24hrs & Pseudomonas Agar Base with CFC supplement at 25 °C for 18-48 hrs (Himedia) for selective the isolation of *Aeromonas*, *Vibrio* and *Pseudomonas* Spp. respectively. *Vibrio* Spp. Yellow and blue centered isolated colony on TCBS agar was inoculated sterile Tryptic Soya Broth (TSB) for further use. Similarly, starch hydrolyzing colony on Bile Salt Brilliant Green Starch Agar (by using Lugol's Iodine) and Fluorescent Colony under the exposure of UV light on Pseudomonas Agar base was inoculated on TSB for further works

Pathogenicity test of isolated organism

In each 3 Aquarium (15 L), five fancy carps of 1 g size was injected intra-dermally with 0.01 ml freshly culture pathogen (10⁻⁴ fold) at the base of dorsal fin. Then observed at the interval of 24, 48 and 96 hrs. Fish mortality record were maintained to study effect of inoculated pathogenic bacterium on fish longevity.

Antibiotics sensitivity testing (AST) profiling

Antibiotics discs with minimum inhibitory concentration (cephalexin 30 mcg, doxycycline 30 mcg, oxytetracycline 30 mcg, Azithromycin 15 mcg, & Amoxicillin 10 mcg of Himedia. Moxifloxacin 5 mcg, Etrapenum 10 mcg, Neomycin 30 mcg, Streptomycin 10 mcg of MAST) with minimum inhibitory concentration (MIC) were tested in vitro using Kirby-Bauer disk diffusion method (Hudzicki, 2016) [17]. Fresh culture of each isolates were prepared on Tryptic Soya Broth then Spread on Muller Hilton Agar plates then antibiotics paper disc was placed aseptically and incubated at 30 °C for 18-24 hrs. Diameter of inhibition was measured using digital Vernier caliper.

Result and Discussions

The study covered the farm from residing as low as 546 masl to the trout farm at 1904 masl where 20% of the farm were public or government owned and rest were private. Almost all the farm had their own backyard sinking pellet feed mill however during survey period 67% of these farm had practice of commercially available feed to feed the stock maintained and 33% had found using farm made pellet feed to fulfill feed requirements. The percentage of farms farm-made pellet feed was found to be driven by seasonal availability and increasing ingredients cost. About 33% of the farms were found to followed recommended stocking density standards and in rest of the farms the stocking density of fingerlings was more driven by availability of seed during stocking period than to water resources and farm carrying capacity. Moreover, all the farm had optimal water quality requirements within trout culture requirements. Also, all of these farm had Tail and fin rot infected fish indicating the disease prevalent and reoccurring between stock cycles. The details of farm history profile and water quality parameters of surveyed farms is shown in Table 1.

Table 1: Trout farming Practices and Tail and fin rot history profile

Farm Code	District	Elevation (MSL, M)	Feed type	Stocking density no's/sq.m	Economic loss,%	Type
R-Dh-G	Rasuwa	1823	Farm-made feed	120	10	Public
R-TB-P1	Rasuwa	1904	Commercial feed	100	12	Private
R-S0-P2	Rasuwa	1918	Commercial feed	210	10	Private
N-TRI-G	Nuwakot	546	Farm-made feed	100	15	Public
N-KK-P1	Nuwakot	1797	Farm-made feed	150	10	Private
N-PK-P2	Nuwakot	1772	Farm-made feed	150	12	Private
L-M-P1	Sindhupalchok	1573	Farm-made feed	150	10	Private
S-ML-P1	Sindhupalchok	1027	Farm-made feed	190	12	Private
S-BC-P2	Lalitpur	1930	Commercial feed	100	15	Public
L-Gd-G	Lalitpur	1530	Commercial feed	100	9	Public
Po-GH-P1	Kaski	1219	Farm-made feed	120	20	Private
Po-GT-P2	Kaski	1359	Farm-made feed	175	15	Private
Po-MP-P3	Kaski	1280	Commercial feed	100	20	Private
Po-IT-P4	Kaski	1222	Farm-made feed	120	15	Private
D-SG-P1	Dhadhing	1167	Farm-made feed	120	12	Private

During the study, water quality parameters as shown in Table 2 ranged as temperature (Tem °C) 8.7-18.2; dissolved oxygen (Do, ppm) 6.9-10; pH 7.2-9.1; total ammonical nitrogen (TAN, ppm) 0.01-0.039; Nitrite (NO₂) 0.01-0.51; nitrate (NO₃, ppm) 0.01-0.17; Total hardness (TH, ppm) 15-105 and Total alkalinity (TAL, ppm) 29-111 in infected raceway ponds. These water quality parameters were within the standard of rainbow trout farming. Moreover, from questionnaire survey, it was found that in case of occurrence of fin and tail rot farmers don't follow the standard protocol to control disease, where 20% of them even do not know

about antibiotics and their resistance to bacteria after repetitive use. The nearby agro vet supplier's recommendation was found to be determinant for the drug, and its dose was found exceeding the earlier maximum limit in each subsequent disease emergence fish batch to control the disease. They are using antibiotics haphazardly, and this malpractice is predominantly prevalent. Three species of gram negative bacteria *aeromonas*, *pseudomonas* and *vibrio* spp. have been reported as major causative agents for tail and fin rot disease in trout (Faruk, Alam, Sarker and Kabir, 2004; Manshadi and Assareh, 2014) [12, 30].

Table 2: Water quality parameters recorded in different farm during fish sampling

Farm code	DO (ppm)	pH	Tem (°C)	TAN (ppm)	NO ₂ (ppm)	NO ₃ (ppm)	TH (ppm)	TAL (ppm)
R-Dh-G	8.2	8.8	11	0.01	0.28	0.17	66	29
R-TB-P1	8.5	8.5	8.7	0.01	0.13	0.01	71	32
R-S0-P2	7.8	8.8	9.9	0.01	0.06	0.01	19	27
N-TRI-G	9.8	8.5	12.1	0.01	0.12	0.01	75	83
N-KK-P1	7.7	9.1	11.6	0.021	0.36	0.05	63	33
N-PK-P2	8.1	7.8	11.3	0.03	0.51	0.01	62	32
L-M-P1	6.9	7.2	11.2	0.02	0.12	0.01	61	29
S-ML-P1	7.3	7.4	11.4	0.03	0.12	0.01	15	29
S-BC-P2	9.6	7.6	11.3	0.028	0.12	0.01	64	64
L-Gd-G	7.9	7.8	14	0.015	0.01	0.02	105	96
Po-GT-P2	7.7	8.3	15.1	0.04	0.49	0.01	90	111
Po-GH-P1	7.9	8.2	14.1	0.04	0.12	0.01	69	79
Po-MP-P3	8.4	7.9	14.1	0.02	0.12	0.02	82	100
Po-IT-P4	7.2	7.9	18.2	0.015	0.32	0.01	98	88
D-SG-P1	10	8.2	15.1	0.01	0.12	0.01	95	87

*D: Diseased, A: Asymptotic as Control; CN: Cephalixin, DO: Doxycycline, N: Neomycin, Mfx: Moxifloxacin and S: Streptomycin Amx: Amoxicillin, Azm: Azithromycin, ETP: Etrapanum and O: Oxytetracycline

All three organisms were isolated from blood, kidney liver and fin swab of diseased fish (Table 3). Among which *aeromonas* spp was more common. And 50% of the asymptotic or control samples from all the 15 farms were also positive for bacteria i.e. those fish samples could have inhabited the dormant tail-fin rot causative pathogens. Isolates from all the samples (diseased and control) were confirmed by biochemical test. Pathogenicity test conformed all the three species were pathogenic as symptoms of erosion of tail and fin was observed in fancy carp fish. With varying degree of pathogenicity. The fish inoculated with *vibrio* and *pseudomonas* showed symptoms of fin erosion after 24 hrs of post inoculation which was earlier than *aeromonas* showing symptoms after 48 hours of post infection. Attempts was made to quantify symptoms on a scale of 0-8 to assign a numerical value. The symptom progressed as Normal fin; White lining on edge of caudal fin; White margins disappears as erosion progress; Fin Fringe begins at edges; Fin erodes

rapidly to its deep layer; Fin erodes rapidly with rupture of blood vessel; blunt tail with no or lesser fin and final death. The difference in pathogenicity among bacteria was found significant ($p \leq 0.05$). The symptoms progressed rapidly in samples inoculated with *vibrio* followed by *pseudomonas* and *aeromonas* bacteria. Moreover, the fish inoculated with *vibrio* remained alive for 7 days after post inoculation, however, those inoculated with *pseudomonas* and *aeromonas* survived for 10 days and 15 days respectively. This indicated that *vibrio* and *pseudomonas* incubation period was rapid enough to slow down immune system of the fish making it more virulence whereas there is slow and chronic progression of disease by *aeromonas* spp. The study on Pathogen and antibiotics resistance profile (Table 3) showed pathogen isolated from all the 15 farms were resistance to Cephalixin, Neomycin, amoxicillin, streptomycin and azithromycin when offered in dose as recommended by NFRC godawari.

Table 3: Pathogen isolated from different farms and its Antibiotics Resistance Profile

Name of Farms	Aeromonas spp%		Vibrio spp%		Pseudomonas spp%		Resistant Antibiotics
	D	A	D	A	D	A	
R-TB-P1	20	15	0	0	25	0	CN, AZM, AMX, S, N
R-Dh-G	33	0	0	0	0	0	CN, AZM, AMX, N
R-S0-P2	20	0	45	5	35	0	CN, AZM, AMX, S, N
N-TRI-G	40	15	10	10	60	25	CN, AZM, AMX, S, N
N-KK-P1	50	40	55	40	20	10	CN, AZM, AMX, S, N
N-PK-P2	100	45	100	70	85	45	DO, MFX, CN, AZM, AMX, S, N, O
L-M-P1	60	55	0	0	85	60	CN, AZM, AMX, S, N, ETP
S-ML-P1	80	75	20	25	28	15	CN, AZM, AMX, S, N, ETP
S-BC-P2	50	30	10	0	25	15	CN, AZM, AMX, S, N
L-Gd-G	0	0	10	10	0	0	CN, AZM, AMX, S, N
Po-GT-P2	70	30	55	30	0	0	CN, AZM, AMX, S, N
Po-GH-P1	55	25	25	15	0	0	CN, AZM, AMX, S, N, O
Po-MP-P3	85	50	50	25	20	20	CN, AZM, AMX, S, N, O
Po-IT-P4	75	30	55	20	20	0	CN, AZM, AMX, S, N, O, ETP
D-SG-P1	25	20	80	45	45	35	DO, MFX, CN, AZM, AMX, S, N, O

Organisms isolated from private trout farm located in dhadhing, D-SG-P1 and nuwakot, N-PK-P2 were resistant to doxycycline, oxytetracycline, moxifloxacin in addition to the antibiotics those were resistant in all the farms. The fish samples collected from these farms also had relatively high microbial load. The most probable reason for these results according to the field survey was the drug being used intermittently continuous within and across production batches. Downstream farm at Nuwakot, N-PK-P2 showed relatively more antibiotics resistance than its upstream farm N-KK-P1. This could be due to of untreated effluents from the upstream is continuously passed to the downstream resulting in variation in antibiotics residues in raceways. Although correlation between antibiotic uses and development of antibiotics resistance was not reported from the survey, the result represented observable misuse of antibiotics. This may be because sampling was not done during clinical outbreak. In farm no N-KK-P1 and N-PK-P2 prescribed antibiotics Florfenicol, Colistin Sulphate are being extensively used. Co-relation between pathogen load and source of water was also observed. The private farms at Kaski Po-GH-P1, Po-IT-P4 and dhadhng D-SG-P1 have relatively higher microbial load when compared to similar size of other farms along with high water temperature and low dissolved oxygen was low and result is supported by earlier study). This illustrates that lower DO and higher temperature is correlated with higher incidence of tail and fin rot (Ajayi and Okoh, 2013; Mosig and Fallu, 2004) [3, 25]. There was also co-relation between pathogen load and source of water. The farms located near the warm spring source had more pathogen load than the farms that were water from flowing systems. Amount of dissolved oxygen may be reason that made the fishes in those farm more susceptible to pathogenic attack. That can also be justified by the fact that R-S0-P2 private trout farm, rasuwa which use water diverted from the streams flowing the mountains have low diseased occurrence, even though with high density stocking. AST (Antibiotic Susceptibility Test), supported the idea of most of the current antibiotics in use being resistant against the pathogens. For instance, even broad spectrum of antibiotics like: cephalexin doxycycline has been found possessing resistance.

Conclusion & Recommendation

In conclusion, the study reveals that trout farms in surveyed fish pocket areas exhibit significant frequencies of bacteria

with low to high level antibiotic resistance. Our results stress the importance of awareness among trout fish farmers in use and misuse of antibiotic along with training to farmers and technical personnel on primary fish health management packages and establishment of mobile diagnostic centers. Furthermore, it highlights the importance of preventive measures in trout farming to minimize the antimicrobial agents as well as their release to effluent water. Further work with increase in sample size, microbial analysis of water for comparison of isolates in inlet and outlet of raceways; molecular characterization of micro flora will help to elucidate the general picture of pathogen and their antibiotics susceptibility pattern nature in trout farms of Nepal.

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