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Isolation of *Flavobacterium branchiophilum* from Rainbow Trout (*Oncorhyncus mykiss*) with Bacterial Gill Disease in Tabriz, Iran

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ABSTRACT: *Flavobacterium branchiophilum* a causative agent of Bacterial Gill Disease (BGD) is a filamentous gram-negative bacteria which produce yellow round colonies in cytophaga agar medium. The outbreak of disease were reported from different part of world. In this study we examined 75 fish sample from farms with a recirculating aquaculture system (n=25), pools with a well water source farms (n=25) and farms with river water supplies (n=25) using cytophaga agar medium for colonies isolation and Gram staining, catalase, oxidase, TSI, SIM, urea, citrate Simon and fermentation tests for identification of colonies. The result shown that 25 samples were infected with *Flavobacterium branchiophilum*. From the 25 cases, 12 were related to the recirculating aquaculture system, 7 to the fish related the river water and 6 to the fish related to well water farms. There is no significance difference between different method of fish culturing was observed (P>0.05). By regarding to our result environmental, management factors and Stressful conditions like crowding, low dissolved oxygen, high ammonia and accumulation may influence the development of BGD in fish farming units.

Keywords: *Flavobacterium branchiophilum*, Rainbow trout, cytophaga agar medium, fish culture systems, Bacterial Gill Disease.

INTRODUCTION

With rapid population growth in developing countries, the need for good nutrition is taken into consideration more and more. Fish meat is considered an excellent choice of meat due to its high levels of omega-3 fatty acids, vitamins and minerals. Rainbow Trout (*Oncorhyncus mykiss*) meat is more palatable to the taste of people due to its high quality and delicious and tasty meat in Iran (Vosoghi and Mostagir, 2000). The increasing development of the fishing industry, intensive culture systems and attention to aquaculture for protein sources must be considered. However, the prevalence and emergence of epidemiologic diseases in the international fishery arena and emergence of various and unknown mortality make the identification of fish pathogens and pollutants necessary and inevitable.

One of the most important diseases that causes morbidity and mortality in the fish farms and decreases production is Bacterial Gill Disease (BGD). The causative agent of disease, *Flavobacterium branchiophilum* is filamentous gram-negative bacteria which produce yellow transparent, round and slime colonies with 0.5-1 mm diameter after 2-5 days after 5 days incubation at 18°C (Boone and Castenholz, 2001; Wakabayashi *et al.*, 1989) (Fig.1).

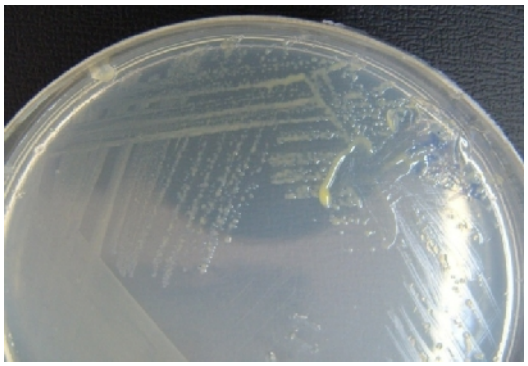

Fig. 1. *Flavobacterium branchiophilum* colonies in cytophaga agar medium.

These species belong to a range of bacteria including the order: Bacteroidetes, class: Flavobacteria, order: Flavobacteriales, family: Flavobateriaceae, and *Flavobacterium* genus (Boone and Castenholz, 2001). The bacteria grow at temperatures between 5-30°C but do not grow at 37°C (Inglis *et al.*, 1993).

Table 1: Identify different species of Flavobacterium (Wakabayashi *et al.*, 1989).

Characteristic	*Flavobacterium branchiophila*	*Flavobacterium aquatile*	*Flavobacterium breve*	*Flavobacterium balustinum*	*Flavobacterium meningosepticum*	*Flavobacterium odoratum*	*Flavobacterium Multivorum*	*Flavobacterium spiritivorum*
Yellow pigmentation	+	+	+	+	D	+	+	D
Growth at 37°C	-	-	D	+	+	+	+	+
Good growth on nutrient agar	-	-	+	+	+	+	+	+
Hydrolysis of:								
Gelatin	+	-	+	+	+	+	d	+
Casein	+	+	+	+	+	+	-	-
Esculin	-	-	-	+	+	-	+	+
Strach	+	NG	-	-	-	-	-	-
Acid produced from:								
Glucose	+	+	D	+	D	-	+	+
Fructose	+	-	-	+	D	-	+	+
Lactose	-	+	-	-	D	-	+	+
Sucrose	+	+	-	-	-	-	+	+
Maltose	+	+	D	-	+	-	+	+
Trehalose	+	-	-	-	D	-	+	+
Cellobiose	+w	-	-	-	-	-	+	+
Arabinose	-	-	-	-	-	-	+	d
Xylose	-	-	-	-	-	-	+	+
Rhamnose	-	-	-	-	-	-	d	-
Raffinose	+w	-	-	-	-	-	+	+
Mannitol	-	-	-	-	D	-	-	+
Salicin	-	-	-	-	-	-	+	+
Indole production	-	-	+	+	D	-	-	-
Nitrate reduction	-	NG	-	-	D	+	-	-
Mol% G+C	29-31	32	32	33	33	31-36	40	41

The bacteria of this family are gram-negative, non-sporza and rod without poly-beta butyrate hydroxy granules and non-motile. Biochemical characteristics of various species of Flavobacteria are shown in Table 1 (Wakabayashi *et al.*, 1989). *Flavobacterium branchiophilum* prefers the diluted nutrient agar and cytophaga agar is commonly used as a medium (Wakabayashi *et al.*, 1989).The disease was first reported by Davis (1926), Ostland *et al.*, (1995) motivate BGD under laboratory conditions. This bacteria grows abundantly 18-24 hours after bacterial suspension on the gill surface in the aquarium medium (Wakabayashi *et al.*, 1989). Poor water quality, stressful rearing conditions and other environmental factors play an important role in colonization of bacteria in gill tissue and produce an outbreak of BGD in fish rearing units (Good *et al.*, 2010; Good *et al.*, 2015).

The contaminated fish become numb, have decreased appetite, and are more interested in staying near the surface of the water or inlet valves. Some modes such as opening the lid gill and coughing may be observed. Respiration rates may increase and mucus secretion may be enhanced so that a continuous layer of mucus is found in fish gills (Ferguson *et al.,* 1991). Infection stimulates the fish's gills and impairs pulmonary function (Wakabayashi and Iwado., 1985). Fish eventually die due to suffocation caused by the high level of dissolved oxygen. Acute infections can be caused losses of 20-50 percent within 24 hours (Wakabayashi *et al.,* 1970). (Fig. 2). Sub-acute form of disease characterized by decrease Na, Cl and protein blood levels, hypoosmolality and increased packed cell volume (Byrne *et al.,* 1995). The infection with *Flavobacterium branchiophilum* can lead to irritation in fish gills and respiratory dysfunction, where eventually the fish die of suffocation due to the high level of dissolved oxygen (Wakabayashi *et al.,* 1985). Ko and Heo (1997) reported bacterial gill disease in cultured rainbow trout in Chung Bok, Korea. They isolated filamentous bacteria, gram-negative with yellow pigment from the gill.

Despite the improvement of fish rearing in Iran and using different culture system, up to now no documented data on the epidemiology of Bacteria Gill disease published. Thus the aim of this study was to evaluate the BGD infection among fish rearing units in Tabriz-Iran.

Fig. 2. A rainbow trout with gill disease. Note the gill rot (arrow).

MATERIAL AND METHOD

The study was carried out during October- December 2009 in Tabriz fish rearing units. 75 samples were collected in order to identify and isolate *Flavobacterium branchiophilum* from rainbow trout gills with bacterial gill disease. For this purpose, a total of 25 samples were taken from fish farms with a recirculating aquaculture system, 25 samples were taken from pools with a well water source and 25 samples of trout fish farms with river water supplies were randomly selected from fishes with gill hyperplasia symptoms associated with lethargy and rapid movements. The collected samples were placed on ice and were transferred to the microbiology laboratory of Islamic Azad University of Tabriz to do the subsequent steps of the research. We used cytophaga agar medium to do the primary gill culture for the fish tingling with bacterial gill disease. This medium is a selected one for Flavobacteria isolation (Wakabayashi *et al.,* 1989; Jooste and Hugo, 1999). After ensuring from the purity of the colonies, they were transferred to a cytophaga broth medium containing 30% glycerin to keep bacteria to continue the research and kept at a temperature of -70°C. Gram staining, catalase, oxidase, TSI, SIM, urea, citrate simon and fermentation tests include glucose, lactose, sucrose, maltose, trehalose, arabinose, xylose, mannitol, Salysyn, raffinose and rhamnose were applied to identify the isolated bacteria.

RESULTS

Of 75 examined samples in this study, 25 cases were isolated as *Flavobacterium branchiophilum* species were confirmed by doing sugar fermentation tests. Also, of the 25 cases, 12 were related to the recirculating aquaculture system, 7 to the fish cultured by the river water and 6 to the fish cultured by well water. There is no significance difference between different method of fish culturing was observed ($P>0.05$).

DISCUSSION

After the first report in Vermont, USA (Davis, 1926), bacterial gill disease was identified as a serious problem throughout North America, Europe and Japan. In Ontario, Canada, bacterial gill disease recognized as one of the most important diseases in salmon farming units (Macphee *et al.,* 1995). They suggest environmental and management factors may influence the development of BGD in fish farming units. Stressful conditions like crowding, low dissolved oxygen, high ammonia and accumulation of particular matter induced BGD in trout (Bullock *et al.,* 1972).

Water quality especially in intensive aquaculture systems also plays an important role in BGD outbreak. One possibility is that feeding produces deteriorating water quality which encourages *Flavobacterium branchiophilum* multiplication on the gill. Also overfeeding may cause proliferation of yellow pigmented bacteria (Snieszko, 1974; Macphee *et al.,* 1995). Feed consumption, digestion and metabolism results increased oxygen consumption, carbon dioxide excretion and excretion of nitrogenous waste, mainly ammonia via diffusion from the plasma, through the lamellar epithelial bilayer (Wright *et al.,* 1985).

In this study The degree of contamination of fish in the recirculating aquaculture systems was high, which could be caused by high levels of ammonia in the water system due to lack of drain water purification, lower water quality and overfeeding in fish farming systems. In contrast with these findings Ferguson *et al.,* (1991) reported that BGD can reproduce in water with good quality, and therefore water quality may only be an exacerbating factor in the development of outbreaks (Good *et al.,* 2010). According to our results most of positive samples were isolated from recirculating aquaculture system and considering that in these systems the water gets back to ponds after a series of processes, the water quality is low in compare pools with well water source and farms with river water supplies, therefore the water quality may play a role in disease distribution.

Elevated fish population in farming ponds associated with fish health and can be a risk factor BGD outbreaks (Good *et al.,* 2010). In the over study density of fish in recirculating aquaculture systems ponds was high and may be of this reason the number of infected fish was high.

Ostkand *et al.,* (1995) isolated gram negative bacteria from *Salmo gairdenri* and *Oncorhynchus masou* infected with bacterial gill disease. These isolate non-motile and form small yellow colonies in cytophaga agar medium in 15-20°C. There also can induce disease in fingerling fish after injection of pure culture of bacteria. In Our isolates also non-motile and form round, smooth and yellow colonies with 0.5-1 mm diameter after 5 days incubation in 18°c.

Wakabayashi *et al.,* (1989), in a study done on 16 bacterial strains isolated from sick fish, introduced *Flavobacterium branchiophilum* species as the causal agent of bacterial gill disease. They used some experiments such as yellow colonies growing, catalase and oxidase tests, indole test and carbohydrate fermentation tests in their study (Wakabayashi *et al.,* 1989). They also commented that the 24-18 hours after exposition of bacteria suspension in aquarium environment there can growth of gill surface. The infection irritates the gills and respiratory dysfunction fish and fish eventually die choking on a high level of dissolved oxygen (Wakabayashi *et al.,* 1989). Ko and Heo (1997) reported bacterial gill disease in cultured rainbow trout in Chung Bok, Korea. They isolated filamentous bacteria, gram-negative with yellow pigment from the gill. The morphological, biochemical, and physiological characteristics as well as the characteristics of isolated antigen strains in Korea were similar to the ones in Japan and the U.S. (Ko and Ho, 1997). The pigments were isolated in this study was forms yellow colonies and consistent with the results of Ko and Heo (1997) and Wakabayashi *et al.,* (1989).

Heo *et al.,* (1990) studied on the incidence of *Flavobacterium branchiophilum* in Tokyo fish farms using culture, light microscope and IFAT methods. There suggested that IFAT method was more specific in compare with two other methods. Also there mentioned since the disease occurs frequently on farms can be concluded that immunity against the disease is not created.

Recent research by Zamora *et al.,* (2013) and Loch *et al.,* (2013) suggests that a plethora of *Falavobacterium* species may be associated, as opportunistic pathogens, with disease in farmed fish, and therefore, with consideration of findings of this article, the further study necessary to further research is necessary to complete our understanding of the ecology of the *Falavobacterium* genus in relation to disease in cultured fishes.

CONFLICT OF INTEREST

The authors declares that there is no conflict of interest regarding the publication of this manuscript.

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