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Research Article

Biotyping and serotyping of *Lactococcus garvieae* isolates in affected farmed rainbow trout (O*ncorhynchus mykiss*) in north Iran

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Abstract

The incidence of Lactococcosis caused by *Lactococcus garvieae,* in rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) farms is disquieting and increasing in recent years. Knowledge of biotypes and serotypes involved in disease incidence is essential to adopt disease prevention and control policies. Twelve isolates of *L. garvieae* from some rainbow trout farms were identified by phenotypic, biochemical, and molecular assays. Biotyping of the isolates was undertaken based on the acidification of carbohydrates including sucrose, tagatose, mannitol, and cyclodextrin as well as the presence of pyroglutamic acid arylamidase (Pyra) and N-acetyl-β-glucosaminidase (β-Nag) enzymes. The Serotyping classification was carried out using slide agglutination assay. The obtained results showed that all isolates were identified as biotype II. Seven isolates (58.3%) were positive for capsule formation and were classified as KG- strain and 41.7 % were classified as KG+ strains (non-capsulated strains). Further works are required for a better understanding of the diversity of *Lactococcus garvieae* isolates in farmed rainbow trout in Iran.

Keywords: *Lactococcus garvieae,* Rainbow trout, Biotyping, Serotyping, Capsule

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Introduction

With the global development of the aquaculture industry, fish lactococcal infections have become an important problem in fish farms of freshwater and marine environments throughout the world (Eyngor *et al.,* 2004; Toranzo *et al.,* 2005; Soltani *et al*., 2015a,b; Lee *et al*, 2020; Radosavljević *et al.,* 2020). Lactococcosis is a prevalent and acute to hyperacute disease in the broad spectrum of fish species caused by Gram-positive cocci, *Lactococcus garvieae* (Tornazo *et al.,* 2005; Meyburgh *et al.,* 2017; Rodrigues *et al.,* 2020). Besides fish, the bacterium was isolated from other animals such as cat, dog, water buffalo, cow, dolphin, prawn as well as food products and planets (Pot *et al.,* 1996; Devriese *et al.* 1999; Barakat *et al.,* 2000; Chen *et al.* 2001; Evans *et al.,* 2006; Kawanishi *et al.,* 2007; Rahman *et al.,* 2020). In addition, lactococcosis considered a zoonotic disease and infected humans with different clinical manifestation of endocarditis, bacteremia, liver abscess, hip prosthetic infection, and meningitis are increasing in recent years, in particular in the regions where people consume raw/semi-raw fish products (Gibello *et al*., 2016; Westberg *et al*. 2020; Rahman *et al.,* 2020).

Rainbow trout as one of the important commercial fish species is very sensitive to lactococosis (Vendrell *et al.,* 2006) and involved in mortality up to 90% (Pereira *et al.,* 2004) especially when the water temperature is above 18°C (Algçet *et al.,* 2009). The affected fish demonstrate symptoms such as anorexia, melanosis, lethargy, erratic swimming, exophthalmia, and mucosal hemorrhage (Rodrigues *et al.,* 2020), and disease outbreaks have been reported from many fish species of both warm water and cold water in freshwater and marine environments worldwide (Chang *et al.,* 2002; Pereira *et al*., 2004; Savvidis *et al.,* 2007; Reimundo *et al*., 2011; Didinen *et al*., 2014; Nelson *et al*, 2016; Ortega *et al.,* 2020).

Because of some differences in biochemical characteristics of *L. garvieae* isolates application of biotyping patterns can be used as a useful tool. The bacterium was categorized into three biotypes of biotype I, II, and III using tagatose and sucrose assays (Eldar *et al.*, 1999). Later, the pathogenic isolates obtained from different animals including fish were proposed into 13 biotypes (biotypes 1 to13) (Vela *et al*. 2000). This assortment was based on the consumption of sucrose, tagatose, mannitol, and cyclodextrin and the presence of pyroglutamic acid arylamidase (Pyra) and N-acetyl-β-glucosaminidase (β-Nag) enzymes (Vela *et al*., 2000). However, minimum data are available on the biotyping of the strains obtained from the diseased fish.

Serological classification of *L. garvieae* isolates are basically correlating with the presence of capsule (Yoshida *et al.,* 1997; Kanai *et al.,* 2017). Two serotypes, KG- serotype (capsulated isolates) that are more virulent (Barnes *et al*., 2002; Ooyama *et*

al., 2002; Kang *et al.*, 2004; Kawanishi *et al*., 2007) and KG+ serotype (non-capsulated isolates) have been defined for *L. garvieae* isolates (Yoshida *et al.*, 1996a; Yoshida *et al.*,1997; Ooyama *et al*., 1999; Kang *et al.*, 2004). In the agglutination test, KG- serotypes are agglutinated with anti-KG- raised serum whereas KG+ serotypes are agglutinated with both anti-KG+ and anti-KG- raised serum (Yoshida *et al.*, 1996a; Barnes *et al*., 2002; Kang *et al.*, 2004; Kawanishi *et al*., 2007). The confirmation for the capsule existence in serological studies is usually performed by electron microscopy scanning or capsule staining methods (Yoshida *et al.*,1996b; Okada *et al*., 2000; Ooyama *et al*., 2002; Pereira *et al*., 2004; Kawanishi *et al*., 2007; Shahi *et al.*, 2018). In recent years, a new serotype has been introduced for some isolates obtained from Japanese marine fish that are unable to agglutinate with anti-KG- sera. These emerging isolates are categorized in serotype II, and the typical isolates agglutinated by anti-KG- sera are classified as serotype I (Ia (KG- strain) and Ib (KG+ strain) (Fukada *et al.*, 2015; Shi *et al.*, 2019).

In Iran, several studies have shown a wide distribution of *L. garvieae* in rainbow trout farms (e.g., Soltani *et al.*, 2005; Soltani *et al*., 2008; Haghighi Karsidani *et al*., 2010; Sharifiyazdi *et al*., 2010; Soltani *et al.*, 2012; Soltani *et al*., 2013; Taherimirghaed *et al*., 2013; Soltani *et al.*, 2015a) but since the adoption of control methods and prevention of disease requires details information on the disease etiology, therefore, this study aimed to study the possible diversity of biotypes and serotypes of *L. garvieae* in Iranian trout farms.

Materials and methods

Sampling and bacteriological study

Samples were collected from 425 moribund fish of 85 rainbow trout farms (at least 5 sick fish from each farm), located in the north of Iran during spring and summer 2018 and 2019. Fish kidney samples were cultured aseptically onto trypticase-soy agar (TSA) (HiMedia, India) incubated at 25°C for 48 h before Gram staining of the grown colonies. The Gram-positive isolates were first identified by phenotyping and biochemical tests including oxidase, catalase, sorbitol utilization on phenol red broth (Quelab, Canada), motility, indole, H_2S production on SIM media (Merck, Germany), urease (Merck, Germany), methyl red, Voges-Proskauer tests on MR-VP broth (Merck, Germany), citrate utilization on Simmon citrate agar (Merck, Germany), and type of hemolysis on 5% sheep blood agar (Merck, Germany) (Soltani *et al*., 2008). Stock cultures of the presumptive isolated identified as *L. garvieae* were maintained at -70°C in trypticase-soy broth (TSB) (HiMedia, India) containing 15% glycerol for further identification by molecular works (Didinen *et al.*, 2014).

DNA extraction and PCR assay

DNA samples were obtained from twelve isolates identified as presumptive

L. garvieae by a Gram-positive genomic DNA extraction kit (MBST Company, Iran). A pair of specific primers shown in Table 1 were used for detection of the 16S rDNA gene and fragment size was 1100 bp for the confirmation of *L. garvieae* isolates (Zoltkin *et al.*, 1998). A volume of 25 µL reaction mixture (7.5µL distilled water, 12.5 µL Mater Mix 2x (Amplicon, Denmark), 1µL of each primer (Sinaclone, Iran), and 3µL of extracted DNA) was used for PCR assay, and Thermocycling conditions included: initial denaturation step for 5 min (94°C), followed by denaturation for 1 min (94°C), annealing for 1 min (55°C), extension for 90 s (72°C), for 35 cycles, and a final extention for 5 min (72°C) using Thermo Cycler (Biorad, USA). Negative control (no template DNA) and positive control were included in each run. The PCR products were electrophoresed using 1.5 % agarose gel at 90v for 90 min and stained with ethidium bromide. The gel was photographed using the Gel Documentation (Genfanavaran, Iran).

Table 1: PCR primers and target gene for molecular identification *Lactococcus garvieae* isolates (Zlotkin *et al*., 1998).

Target gene	Primer name	Primer set	Amplicon size
16S-rDNA	pLG-1 (F)	5'-CATAACAATGAGAATCGC–3'	1100bp
	pLG-2 (R)	5'-GCACCCTCGCGGGTTG–3'	

Biotyping assay

Biotying of *L.garvieae* isolates was performed based on the acidification of four sugars including sucrose, tagatose, mannitol, and cyclodextrin, as well as the presence of two enzymes, including pyroglutamic acid arylamidase (Pyra), and N-acetyl-β-glucosaminidase (β-Nag) (Vela *et al.*, 2000).

For sugar acidification tests, one gram of each mentioned sugar (all from Sigma, USA) was first dissolved in 100 mL of distilled water before being filtered by 0.22 µm Millipore filter into a sterile container. Sterile phenol red broth (Quelab, Canada) was added 5 ml into tubes, before adding five drops of each sugar. A loop of bacterial colony grown on TSA (HiMedia, India) was inoculated into the tubes incubated at 30°C for 24 h and the results were read based on the color change (production of acid changes the color to yellow).

The activity of the enzyme Pyra was carried out by growing *L. garvieae* colonies into PYR broth medium (Quelab, Canada) at turbidity equal to MacFarland standard No 2 incubated at 35°C for 3 h. One drop of Dimethylaminocinnamaldehyde Sigma, USA) reagent was then added and the results were considered as positive if dark color (purple red) appeared within 2 min (if the color changed to pink, orange, or yellow the test was negative) (Hébert *et al.*,1988).

The test for β-Nag activity was undertaken by growing the bacterial colonies into tubes containing TSB (HiMedia, India) incubated at 30°C for 24 h. The tubes were centrifuged at 5000 rpm for 10 min and the bacterial cells

were suspended into 10 mM sterile phosphate-buffered saline (PBS), dispensed into microtubes, and centrifuged at 10000 rpm for 5 min. The bacterial cells were re-suspended into 25 mM PBS and adjusted at optical density (OD) equal to 0.1 at 546 nm with spectrophotometry (UNICO, China). Chromogenic substrate 4-Nitrophenyl N-acetyl β-D-glucosaminide (Sigma, USA) was prepared in PBS at a concentration of 10 mM (final concentration of substrate was 10 mM prepared in PBS as solvent). A volume of 60 µl of each bacterial suspension and substrate was mixed, incubated at 37°C for 4 h, and the results were considered positive if the color changed from colorless to yellow (Kadyan *et al.*, 2020).

Serotyping assay

Anti-sera preparation

Colonies of 24-h fresh cultures of the confirmed bacterial isolates of *L. garvieae* were transferred to sterile tubes containing 5 ml PBS equal to MacFarland No 0.5. The isolated were inactivated at a 90°C water bath for 90 min., and the inactivated cell suspensions were stored at 4°C until used for the assay. The safety of the heat-killed cells was also checked by inoculation of 0.1 ml of each inactivated cell suspension on TSA at 30°C for up to 72 h.

To produce anti-sera to the isolates of *L. garvieae*, twenty-four healthy rats (200-220 g) divided into 12 groups (each group two rats) in separate cages were injected intraperitoneally with 100 µl/rat of the heat-inactivated whole cells of the bacterial isolates followed by two boosters at 10 days intervals. The hyper-immune rats were anesthetized before the blood samples being obtained from their hearts, and the sera were obtained after the blood samples were centrifuged at 3000 rpm for 5 min.

Agglutination test

Agglutination assay was carried out using slide agglutination by mixing a droplet of each anti-serum sample with a colony of the fresh culture of each bacterial isolate performed on a clean slide, and the results of agglutination were read after 30 s and 180 s under microscopy with low magnification. The level of agglutination was considered as high, moderate, low, and negative (Table 4). The reaction between homologous anti-sera samples and the bacterial isolates was considered as control positive, while the reaction between the sera samples and sterile PBS was considered as control negative.

Capsule staining

Capsule staining assay was carried out using the method described by Colwell and Grigorova (1987). A small droplet of Indian ink was put on the slide and a needlepoint number of each bacterial isolate grown on TSA was mixed in, covered with a coverslip, and was examined under compound microscopy at 100 × magnification.

Results

Affected fish exhibited various signs including darkening of body, bilateral exophthalmia, cataract, melanosis, and abdominal distension and, in some cases eye explosion and anorexia were seen (Fig. 1). The mortality rates were varied from 30 to 60%. In most fish farms water temperature exceeded 18°C due to the summer season.

Figure 1: Affected rainbow trout collected for the study. A. Hemorrhage in periorbital and intraocular area, B. Abdominal distension with ascetic fluid, C. Exophthalmia and darkening of skin, D. Hemorrhage on surface of liver.

Phenotypic and molecular results

From 78 bacterial isolates recovered from the diseased fish, 22 isolates (28.2%) were Gram-positive, and 56 isolates (71.8%) were Gram-negative. Phenotypic and biochemical characterization of the Gram-positive strains resulted in presumptive identification of 12 (54.55%) isolates as *L. garvieae* isolates and the rest as *Streptococcus* sp. (45.45%, 10 strains). These isolates were Gram-positive cocci, α-hemolytic (Fig. 2), oxidase negative, catalase negative, sorbitol positive, non-motile, indole negative, H_2S production negative, urease negative, methyl red positive, and Voges-Proskauer negative.

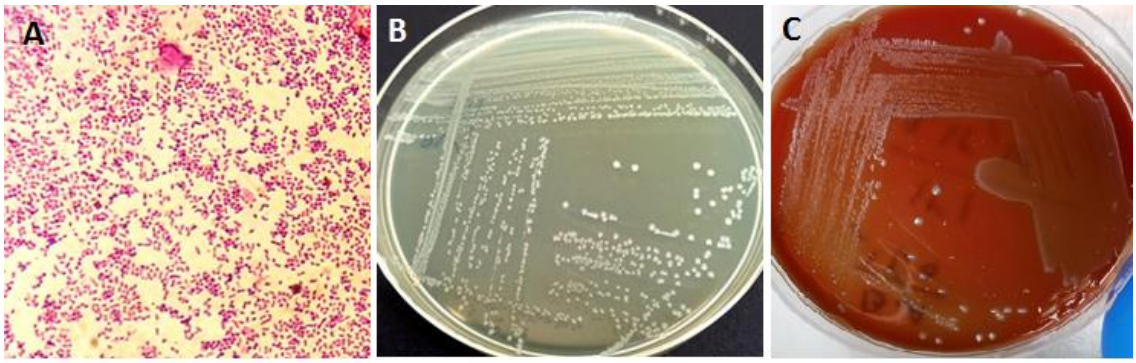

Figure 2: Phenotypic characterization of *L. garvieae* A. Microscopic view of Gram-positive cocci. B. growth whitish to grayish colony on TSA. C. α-hemolysis on blood agar.

All isolates also were negative for citrate assay except two isolates (isolates 41, 73) that were negative. In the PCR assay, all 12 isolates were identified as *L. garvieae* with a molecular weight of 1100 bp identical to the positive control i.e., *L. Garvieae* (Fig. 3). The primary characterization of the rest recovered Gram-negative strains were members of genera *Yersinia* sp. (62.5%), *Aeromonas* sp. (23.21%) and *Citrobacter* (14.28%) (Table 2).

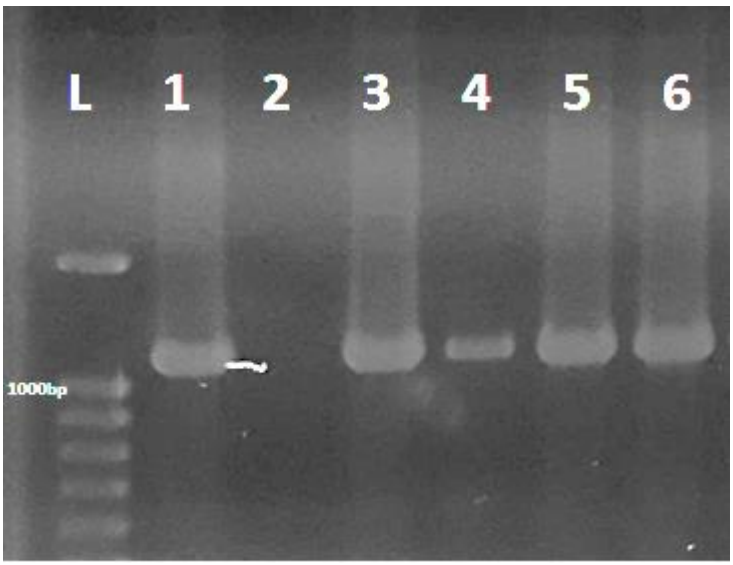

Figure 3: Identification of *L. garvieae* by PCR. Lane L: DNA ladder 100 kb; Lane 1: positive control (1,100 bp); Lane 2: negative control; Lane 3-6: positive samples for *L.garvieae*.

Bio-typing assay

In the biotyping assay, all twelve isolates of *L. garvieae* were positive for acid production from tagatose, sucrose, mannitol, and cyclodextrin (Fig. 4). Also, all isolates were positive and negative for the presence of Pyra and β-Nag enzymes, respectively identical to isolates of biotype 2 (Table 3).

Table 2: Biochemical characteristics of bacteria recovered from the diseases rainbow trout and characterized at genus level.

Character	*Yersinia* sp.	*Aeromonas* sp.	*Citrobacter* sp.	*Streptococcus* sp.
Gram staining	- rod	- rod	- rod	+ cocci
Oxidation/fermentation	+/+	+/+	+/+	
Motility at				
24°C	+	+	+	-
30°C	+	+	+	-
Catalase	+	+	+	-
Hemolysis				α/β
Cytochrome-oxidase	+	+	-	-
ONOG	+	+	+	
Arginine-dehydratase	-	+	-	
lysine-decarboxylase	+	+	-	
Ornithine-decarboxylase	+	-	-	
Citrate	V	+	+	-/+
H2S	-	-	+	-
Urease	-	-	-	-
Tryptophane deaminase	-	-	-	
Indole	-	+	-	-
Voges Proskauer	-	+	-	-
Gelatinase	-	+	-	
Glucose	+	+	+	
Mannitol	+	+	+	
Inositol	-	-	-	
Sorbitol	-	-	+	
Rhamnose	-	-	+	
Saccharose	-	+	-	
Melibiose	V	-	-	
Amygdalina	-	+	-	
Arabinose	-	-	+	
Methyl red	+	+	+	+
Aesculin hydrolysis	-	+	+	
Triple sugar iron	K/A	K/A	K/A	

Figure 4: A. Acidification of sugars. Acid production in sample tube (right one) turned the color of media from red to yellow, left one was negative control. B. PYR test: dark red color of first row (all of them were samples) showed positive results, second row was negative control with orange color. C. β-Nag test: left microtube was sample and right was negative control without any color changes.

Serotyping studies

All antisera exhibited a strong agglutination reaction with homologous isolates, but different levels of agglutination from negative (-), weak (+), moderate (++), and strong (+++) agglutination with other isolates (Table 4). In capsule staining assay seven isolates (isolates 65, 121, 201, 1323, 21, 41 and 73) were capsulated (KG- serotype), while five isolates (isolates 124, 46, 71, 72 and 1061) were non-capsulated (KG+ serotype) (Fig. 5). In most cases, sera raised against non-capsulated isolates only agglutinated non-capsulated isolates, but sera raised against capsulated isolates agglutinated both capsulated and non-capsulated isolates (Fig. 6).

Table 3: Results of biotyping of *L. garvieae* isolates obtained from rainbow trout. Sac = sucrose, Tag = tagatose, Man = mannitol, Cedex = cyclodextrin, Pyra = pyroglutamic acid arylamidase, β-Nag = N-acetyl-β-glucosaminidase.

L. garvieae isolates	Level of agglutination												Capsule	Serotype
	124	46	71	72	1061	65	121	201	1323	21	41	73		
124	+++	++	++	+	++	+	++	+	++	++	++	++	-	KG+
46	++	+++	++	++	++	++	++	++	+	++	++	++	-	KG+
71	++	++	+++	+++	+	+	++	++	++	++	+	+	-	KG+
72	++	+++	+++	+++	++	++	++	++	++	+++	++	+++	-	KG+
1061	++	++	++	++	+++	++	++	++	++	++	++	++	-	KG+
65	-	-	-	-	-	+++	++	++	++	+	+++	++	+	KG-
121	-	-	-	-	-	++	+++	++	++	++	+++	+++	+	KG-
201	-	-	-	++	-	+	++	+++	+	+	+	+++	+	KG-
1323	-	-	++	-	-	+	+	+	++	+	-	+	+	KG-
21	-	-	-	-	-	-	-	+	-	++	-	++	+	KG-
41	-	-	-	-	-	+	+	++	++	++	++	+	+	KG-
73	-	-	-	++	-	++	++	++	++	++	++	+++	+	KG-

Table 4: Slide agglutination results between antiserum and isolates of *L. garvieae.* Negative (-), weak (+), moderate (++) and strong (+++) agglutination.

Bacterial isolates	Biochemical test						Biotype
	Sac	Tag	Man	Cedex	Pyra	β-Nag	
124	+	+	+	+	+	-	2
46	+	+	+	+	+	-	2
71	+	+	+	+	+	-	2
72	+	+	+	+	+	-	2
1061	+	+	+	+	+	-	2
65	+	+	+	+	+	-	2
121	+	+	+	+	+	-	2
201	+	+	+	+	+	-	2
1323	+	+	+	+	+	-	2
21	+	+	+	+	+	-	2
41	+	+	+	+	+	-	2
73	+	+	+	+	+	-	2

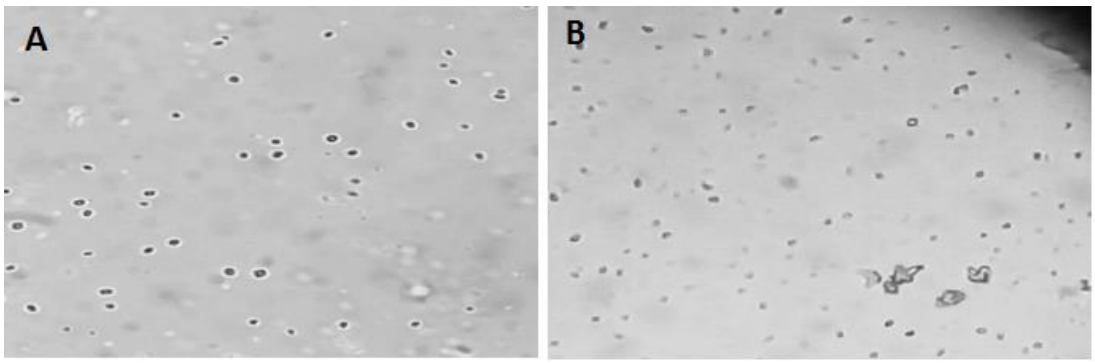

Figure 5: (A) Positive capsule and (B) Negative capsule isolates of *L. garvieae* obtained from diseased trout in this study.

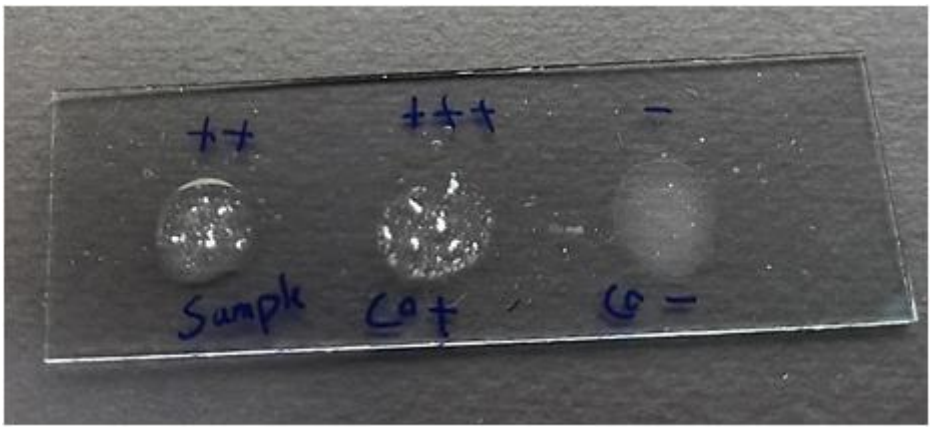

Figure 6: Slide agglutination test. (+++)=strong agglutination (positive control), (++)= moderate agglutination (sample), (-)=no agglutination (negative control).

Discussion

Different studies analyzed the biochemical characteristics of *L. garvieae* isolates obtained from different animals including aquaculture species. For instance, based on the acidification of sugars, three biotypes of the bacterium have been reported by Elder *et al.* (1999), while Vela *et al.* (2000) described 13 biotypes from different animals including trout, cow, buffalo, water, human, and yellowtail which six biotypes were obtained from trout. These authors suggested a new scheme based on the acidification of four carbohydrates including sucrose, tagatose, mannitol, and cyclodextrin, and the production of two enzymes i.e., pyroglutamic acid arylamidase and N-acetyl-β-glucosaminidase. Later, in a biochemical study conducted by Ture *et al.* (2015), 42 isolates of *L. garvieae* collected from Turkey, Spain, France, and Italy were categorized into twenty biotypes. In addition, it has been demonstrated that the bacterial isolates in a specific region may present identical phenotypic and biochemical features as fourteen *L. garvieae* isolates from Mexican rainbow trout farms exhibited similar biochemical patterns representing only as biotype II isolates (Ortega *et al*., 2020). Here in the present study, we found that these 12 strains of *L. garvieae* isolated from different trout farm revealed a similar homogeneity of

biochemical features, suggesting that the isolates of a local region e.g., Mazandaran province may have high homogeneity in biochemical features as biotyping assay exhibited that all isolates are classified in biotype II. However, more studies are required to assess such homogeneity of the bacterium in other states of Iran.

Serological classification of *L. garvieae* isolates is differentiated with ability or inability of KG+ antiserum to agglutinate the studied isolates which is directly related to presence or absence of capsule surrounding the isolates and based on this present work, *L. garvieae* isolates can be divided into two serotypes of KG- (capsulated) and KG+ (non-capsulated). The KG- serotype was agglutinated only by KG- antiserum while the KG+ serotype could be agglutinated with both KG+ and KG- antisera (Yoshida *et al.*, 1996a; Yoshida *et al.*,1997; Ooyama *et al*., 1999; Kawanishi *et al*., 2007). Our results showed that five recovered isolates of *L. garvieae* from trout were classified as serotype KG+, while seven isolates were KG-. It has been shown that the geographical origin of some capsulated isolates of *L. garvieae* may affect the agglutination assay as the antisera raised against some capsulated isolates may be unable to agglutinate the capsulated isolates with far origin from different continents and thus, according to this, KG- serotypes have been divided to two sub-classes (Barnes and Ellis, 2004). Even Japanese serotypes I could be categorized into serotypes Ia (KG- or capsulated strain) and Ib (KG+ or non-capsulated strain) that are agglutinated with anti-serum raised to capsulated strains. However, Japanese serotypes II are unable to give an agglutination reaction with anti-serum raised to capsulated strains, suggesting more studies are required associated with the serotyping of *L. garvieae* strains as the cause of disease outbreaks in finfish (Fukada *et al.*, 2015; Shi *et al.*, 2019).

One of the interesting findings in our study was that 58.3% of these *L. garvieae* isolates were KG- serotype (capsulated strains) able for capsule production, suggesting the significant role of the capsule as one of the major virulence factors involved in the bacterial pathogenesis (Wu *et al*., 2008). Therefore, as capsule positive bacterial pathogens are relatively more resistant to environmental fluctuations such as temperature, pH, and salinity (Corona-Hernandez *et al.*, 2013), thus, it seems the positive capsule strains may play more role in outbreaks of lactococcosis caused by *L. garvieae* in Iranian trout farms, although more epidemiological works are required to confirm it. Capsule formation also is a good tool to protect the bacteria against the host immune responses in various ways such as prevention of phagocytosis (Meyburgh *et al.*, 2017), indicating why the rate of morbidity and mortality by *L. garvieae* is raising in Iranian trout farms as the capsular strains of *L. garvieae* are more virulent than non-capsular strain (Barnes *et al.*, 2002; Ooyama *et al*., 2002; Kang *et al.* 2004; Kawanishi *et al*., 2007;

Morita *et al.,* 2011). It is, therefore, worth mentioning that the use of capsulated strains for protection routes such as immunization is more efficacious as indicated by some researchers such as Ooyama *et al.* (2002).

In conclusion, all *L. garvieae* isolates causing lactococcosis in rainbow trout farms in Mazandaran province were biotype II but with 41.7% as KG+ serotype (non-capsulated strains) and 58.3% as KG- serotype (capsulated strains) indicating a predominant role of capsulated in morbidity and mortality in trout farms in north Iran. More studies are, however, required to show the level of homogeneity of *L. garvieae* in trout farms in other states of the country. Such studies can lead to the production of a vaccine to cover satisfied protection against the disease in Iranian trout farms located in different regions.

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