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Biotyping of *Lactococcus garvieae* Isolated from Turkey

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Özet: T*ürkiye'den izole edilen* Lactococcus garvieae*'ların biyotiplendirilmesi.* Alabalıklardan izole edilen 20 *Lactococcus garvieae* suşu fenotipik özellikleri, serolojik özellikleri ve kapsül oluşumu yönünden incelendi. İzolatlar API 20 Strep API 50 CH ve klasik bakteriyolojik yöntemlerle identifiye edildi. Suşların tamamının kapsullü olduğu, kapsullü İspanya suşuna karşı hazırlanmış antiserum ile aglutine olduğu görüldü. Sonuçlar Türkiyeden yapılan izolatların homojen ve biyotip 3 olduğunu, ve İspanyadan yapılan izolatlatlarla biribirine yüksek oranda benzediğini ortaya koydu. Klasik bakteriyolojik incelemede izolatların %40'ının laktozdan asit oluşturabildiği belirlendi.

Anahtar Kelimeler: *Lactococcus garvieae*, biyotip, serotip, kapsül, alabalık.

Abstract: Twenty *Lactococcus garvieae* strains isolated from rainbow trout were investigated in phenotypic, serologic and capsule formation. Isolates were identified with API 20 Strep, API 50CH and conventional bacteriologic techniques. All strains were capsulated and agglutinated with antiserum, which raised against capsulated Spanish strain. The results were indicated that high level of homogeneity in Turkish isolates, all were biotype 3 and high similarity between Spanish and Turkey isolates. 40% of the isolates were able to produce acid from lactose in conventional techniques

Key Words: *Lactococcus garvieae*, biotype, serotype, capsule, rainbow trout.

Introduction

Gram positive, oxidase and catalase negative, non motile cocci, which isolated from fish was considered in genus *Streptococcus* (Plumb *et al*., 1974; Booker *et al*, 1979; Bragg and Broere 1986; Kim and Lee, 1994) *Enterococcus* (Kusuda *et al*., 1991) *Vagococcus* (Walbanks *et al*., 1990) and *Lactococcus* (Williams *et al*., 1990 Prieta *et al*., 1993). Nevertheless some of the isolates were thought not belong to any of the above genus but they were closed to Enterococcus (Carson *et al.*, 1993; Toranzo *et al.*, 1994). It has clearly understood that previously Enterococcus-Like bacteria named pathogen is *Lactococcus garvieae* and biochemical characteristics, protein profile, 16S rRNA sequencing, and DNA hybridisation studies confirmed that *L. garvieae* and *E. seriolaicida* are the same species (Domenech *et al.*, 1993; Eldar *et al*., 1996; Teixeira *et al.*, 1996). It causes hemorrhagic septicaemia, enteritis, ascites, bilateral exophthalmus with haemorrhage; darkening of the skin; congestion of the intestine, liver, kidney, spleen, and brain; and hemorrhagic enteritis in farmed trout (Dome´nech *et al.*, 1993; Cagirgan and Tanrikul, 1995; Salati *et al*., 1996; Diler *et al*., 2002). Lactococcosis cause more then 50% mortality in rainbow trout in summer season when the water temperature exceeds 15°C. The treatment of the disease is not successful with chemotherapeutics because of development of the resistance and recurrent infection. The vaccination seems only available solution against the disease. (Ghittino and Prearo, 1993). However choosing of the strain to produce vaccine depends on the characteristics of the antigen.

Eldar *et al.*, (1999) was reported three biotypes, which based on acidification of sugars and nine ribotypes. However Vela *et al.*, (2000) have differentiated 13 biotypes and 19 pulsotypes based on acidification of saccharose, tagatose mannitol cyclodextrin and presence of pyroglutamic acid arylamidase (Pyra) and N-acetyl-ß-glucosaminidase (ß-Nag) in *L. garvieae* which isolated from rainbow trout, yellow tail, cow, buffalo and human from different countries including Spain, Portugal France Italy Japan USA and Brazil. Serological characteristics and immunogenicity of the *L. garvieae* depends having capsule in developing immunity (Barnes *et al.*, 2002). However, there is no information on *Lactococcus garvieae* biotypes and capsule formation as well as agglutination properties of the isolates from Turkey. In this research 20 *L. garvieae* were biotyped using API 20 Strep and API 50 CH tests conjunction with conventional bacteriology techniques. Besides biotyping, agglutination and capsule formation were tested.

Materials and Methods

Twenty strains were subjected to research. 19 of *L. garvieae* strains, which isolated from diseased rainbow trout from Turkey between 1999 and 2003 One was isolated in 1995. They were stored in 15% glycerol containing tryptic soy broth at -20°C until determine biochemical profile. Two Spanish isolates are kindly provided by Dr. Toranzo. NCDO 2155 is also included as reference strain. All the strains were plated out and purified on 5% defibrinated sheep red blood cell containing trypticase soy agar (TSA+B) incubating aerobically at 24±0.5°C.

API 20 Strep test and API 50 CH tests (Biomerieux, France) were used to determine biochemical characteristics of bacteria according to the manufacturer instruction except incubation temperature, which was 24±0.5°C. All strains were spectrophotometrically adjusted (Optic density 0.8 at 580 nm wave length) inocula prepared from TSA+B grown colony. Blood degradation was determined on TSA+B incubating aerobically at same temperature incubating for 2 days (Falclam and Elliott, 1995). For catalase test one drop of 3% hydrogen peroxide was mixed with Trypticase soy agar (TSA) (Caso agar, Merck) grown colony on a slide. Oxidase test was performed with TSA grown colony streaking to Merck Strip (Merck) growth at pH 9.6 was tested in brain hearth infusion broth (BHIB, Merck) Salt tolerance tests were performed in 6.5% NaCl and bromcresol purple (BCP) containing brain hearth infusion broth (BHIB, Merck) incubating up to 14 days. Oxidation and fermentation test (OF) were carried out in modified OF medium (Koneman *et al.*,1992; Falcklam and Elliott, 1995) Growth at 10°C and 45°C were tested in 0.1% dextrose and BCP added BHIB. Acid formation from lactose, sorbitol, D-arabinose, melibiose, rhamnose, inositol, inuline were also determined in BCP and sugar added BHIB incubating up to 2 weeks according to the method of Faclam and Elliott (1995). Voges proscourer (VP) and methyl red (MR) were tested in VP-MR medium (Merck).

Slide agglutination tests were carried out with all isolated strains using raised rabbit serum against capsulated Spanish isolate (279) (Toranzo *et al.*, 1987). Capsule formation was also determined by the method of Prof. J. Romalde (Personal communication). One TSB+B grown colony suspended in PBS (pH 7.4) and fixed with equal volume of fixing solution (2.5% glutaraldehyde containing 100 mM L-lysine HCL, Merck) for 20 minutes at room temperature. A loopfull suspension put on a slide and air-dried. Then stained with 3 minutes with basic fuchsine and investigated with light microscope. Red stained bacteria which surrounded by unstained halo is capsule positive.

Result and Discussion

Lactococcosis is causing high level of mortality in many countries (Austin and Austin, 1999) While Vela *et al.*, (2000) were reporting 13 biotypes of *L. garvieae*, which isolated from different countries and species, Eldar *et al.* (1999) were reported three biotypes. Spanish strains were Biotype 3. In this research all strain were Gram positive 2-10 ovoid cocci containing chains, α haemolytic on TSA+B. Oxidase, catalase, gelatine degradation, H_2S production, urea degradation, indol production, nitrate reduction and motility tests were negative. O/F test were positive and sensitive to O/129, and were able to growth in 6.5% NaCl containing medium as well as at pH 9.6. All strains were positive in VP when tested in API 20 Strep test, lately positive (2-4 days) in conventional techniques at same temperature. Acid production from galactose, amygdaline (Weak in API 20 Strep, strong positive 50 CHL), D-tagatose, gluconate were weakly positive. API profiles of Spanish strains were the same with Turkish strains. 8 strains (40%) were produced acid from lactose in 7 days incubation following the method of Faclam and Elliott, (1995). 96% of Spanish strains and 20% of Taiwan were produced acid from lactose in conventional technique when blood agar grown colony tested (Ravelo *et al.*, 2001; Chang *et al.*, 2002) The phenotypic characteristics of isolated *Lactococcus garvieae* strains were tabulated in Table 1.

Table 1. Penotypic characteristics of *Lactococcus garvieae* isolated from Turkey.

Phenotypic tests		Phenotypic tests	
Catalase	- (20/20)	L-Sorbose	-(20/20)
Oxidase	- (20/20)	L-Rhamnose	-(20/20)
H_2S	- (20/20)	Dulcitol	-(20/20)
Urease	- (20/20)	Inositol	-(20/20)
Citrate	- (20/20)	D-Mannitol	+(20/20)
Nitrate	- (20/20)	D-Sorbitol	-(20/20)
Motility	- (20/20)	Methyl-αD-Mannopyranoside	-(20/20)
Indol	- (20/20)	Methyl-αD-Glukopyranoside	-(20/20)
O/F test	+(20/20)	N-Acetyl glucosamine	+(20/20)
Growth in 6.5 NaCL	+(20/20)	Amygdaline	+(20/20)
Growth in pH 9.6	+(20/20)	Arbutin	+(20/20)
Growth at 10 °C	+(20/20)	Esculin	+(20/20)
Growth at 45 °C	+(20/20)	Salicin	+(20/20)
Gelatin degradation	- (20/20)	D-Cellobiose	+(20/20)
Hemolysis	α	D-Maltose	+(20/20)
VP	+ (20/20)	D-Lactose	-(20/20)
MR	+ (20/20)	D-Lactose (Conventionel)	+(8/20)
Hippurat	+ (20/20)	D-Melibiose	-(20/20)
Pyra	+ (20/20)	D-Saccharose	+(20/20)
α galactosidase	- (20/20)	D-Trehalose	+820/20)
β Glukorinidase	- (20/20)	Inuline	-(20/20)
β Galactosidase	- (20/20)	D-Melezitose	-(20/20)
Alkaline phosphotase	- (20/20)	D-Raffinose	-(20/20)
Leucine arylamidase	+(20/20)	Amidon	-(20/20)
ADH	+(20/20)	Glycogene	-(20/20)
Glycerol	-(20/20)	Xylitol	-(20/20)
Erythritol	-(20/20)	Gentiobiose	+(20/20)
D-Arabinose	-(20/20)	D-Turanose	-(20/20)
L-Arabinose	-(20/20)	D-Lyxose	-(20/20)
D-Ribose	+(20/20)	D-Tagatose	+(20/20)
D-Xylose	-(20/20)	D-Fucose	-(20/20)
L-Xylose	-(20/20)	L-Fucose	-(20/20)
D-Adonitol	-(20/20)	D-Arabitol	-(20/20)
Methyl-βD-xylopyranoside	-(20/20)	L-Arabitol	-(20/20)
D- Galactose	+(20/20)	Potasium Gluconate	+(20/20)
D-Glucose	+(20/20)	Potasium 2-Ketogluconate	-(20/20)
D-Fructose	+(20/20)	Potasium 5-Ketogluconate	-(20/20)
D-Mannose	+(19/20)		

All strains produced acid from sucrose, tagatose, mannitol, gluconate and Pyra, VP MR reactions were positive. Italian strains did not produce acid from either sucrose or tagatose (Vela *et al.*, 2000). All isolated strains in this research were reduced hippurat in contrast to Italian Australian and Japanese isolates (Eldar *et al.*, 1999). Isolates from Taiwan non-haemolytic, O/F non-fermentative, some of the strain produced acid from α-methyl-D-glucoside, melesitose, glycogen and L-arabitol (Chang *et al.*, 2002). O/F test is important to differentiate between micrococci and streptococci or staphlococci. However, some modification should be done in OF medium for cocci (Coneman *et al.*,1992).

In this research, all of the isolated *L. garvieae* strains

were positive in slide agglutination using rabbit serum, which raised against capsule positive Spanish strain 279. All strains in this study were positive in capsule formation in contrary to Japanese strains (Hirono *et al.*, 1999) Two different phenotypic profiles were determined in acid production from lactose in conventional technique. The results showed that isolated strains from Turkey are very similar to Spanish strains and Biotype 3.

References

Austin, B., D. A. Austin, 1999. Bacterial fish pathogens: Diseases of Farmed and Wild Fish, 3nd rev.ed. Praxis Publishing Ltd, Chichester, UK.

Barnes A. C., C. Guyot, I., B. G. Hansen, K. Mackenzie, M. T. Horne, A. E. Ellis, 2002 Resistance to serum killing may contribute to differences in the abilities of capsulate and non-capsulated isolates of *Lactococcus garvieae* to cause disease in rainbow trout.*(Oncorhynchus mykiss* L.).Fish&Shellfish Immunol. 12: 155-168.

Booker, J., G. D. Jr. Imes, C. M.Cameron, T. W.Naude, H. J. Schoonbee, 1979. Trout Mortalities as a result of *Streptococcus* Infection. Onderstepoort. J.Vet. Res. 46, 71-77.

Bragg, R. R., J. S. E. Broere, 1986.Streptococcosis in Rainbow Trout in South Africa.Bull. Eur. Ass. Fish Pathol. 6 (3) 89-91.

Cagirgan, H., T. T. Tanrikul, 1995. A new problem Enterococcus-like Infection in Rainbow Trout (*Oncorhynchus mykiss*) Farms in Turkey (in Turkish). Vet. Kont. Ve Arast. Enst. Md. Derg. C. 19, S.33: 9-19.

Carson, J., N. Gudkovs, B. Austin, 1993 Characteristics of an *Enterococus* like Bacterium from Australia and South Africa, Pathogenic for Rainbow Trout, *Oncorhynchus mykiss* Walbaum. J.Fish Dis. 16: 381-388.

Chang, P. H., C. M. Lin, H. Plarre (2002) *Lactococcus garvieae* infection of cultured rainbow trout, *Oncorhynchus mykiss*, in Taiwan and associated biophysical characteristics and histopathology. Bull. Eur. Ass. Fish Pathol., 22(5)319-327.

Diler, Ö., S. Altun, A. K. Adiloğlu, A. Kubilay, B. Işıklı, (2002) First Occurance of Streptococcosis affecting farmed rainbow trout (*Oncorhynchus mykiss*) in Turkey. Bull. Eur. Ass. Fish Pathol 22(1) 21-26.

Domenech, A., J. Prieta, J. F. Ferna´ndez-Garayza´bal, M. D. Collins, D. Jones, L. Domý´nguez. 1993. Phenotypic and phylogenetic evidence for a close relationship between *Lactococcus garvieae* and *Enterococcus seriolicida*. Microbiologia 9: 63–68.

Eldar, A., C. Ghittino, L. Asanta, E. Bozzetta, M. Gloria, M. Prearo, H. Bercovier, 1996. *Enterococcus seriolicida* is a junior synonym of *Lactococcus garvieae*, a causative agent of septicemia and meningoencephalitis in fish.Curr. Microbiol. 32: 85–88.

Eldar, A., M. Gloria, C. Ghittino, A. Zlotkin, H. Bercovier. 1999. Biodiversity of *Lactococcus garvieae* strains isolated from fish in Europe, Asia, and Australia. Appl. Environ. Microbiol. 65: 1005–1008.

Faclam, R., J. A. Elliott (1995). Identification, Classification, and Clinical Relevance of Catalase-Negative Gram-Positive Cocci, Excluding the Streptococci and Enterococci. Clin. Microbiol. Rew.8: 479-495.

Ghittino C, M. Prearo (1993). Comparison of some strains isolated from rainbow trout affected by streptococcosis. Boll. Soc. Ital. Patol. 11:30-43.

Hirono I., H. Yamashita, C. Park, T. Yoshida, T. Aoki, 1999. Identification of genes in a KG- phenotype of Lactococcus garvieae, a fish pathogenic. Microbial Pathogen. 27: 407–417.

Kim, Y. G, K. K. Lee, 1994. Isolation Characterisation and Pathogenicity of a *Streptococcus* in the Flounder (*Paralichthys olivaceus*) Cultured in Korea. Bull. Eur. Ass. Fish Pathol. 14 (1), 8-11.

Koneman, E. W., S. D. Allen, W. M. Janda, P. C. Schreckenberger, W.C. Jr. Winn, 1992. Color Atlas and Textbook of Diagnostic Microbiology Fourth Ed. p.431-466.

Kusuda, R., K. Kawai, F. Salati, C. R. Banner, J. L. Fryer, 1991. *Enterococcus seriolocida* sp. nov., a Fish Pathogen. Int. J. Syst. Bacteri. 41, 406-409.

Mundt, J. O., 1986 Lactic acid streptococci, pp.1065-1066. In Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. (Eds) Bergey's Manual of systematic Bacteriology, Vol 2.Williams and Wilkins, Baltimore.

Plumb, J. A., J. H. Schachte, J. L. Gaines, W. Peltier, and B. Carol, 1974.*Streptococcus* sp. from marine fishes along the Alabama and North-West Florida coast of Gulf of Mexico. Trans. Am.Fish. Soc.103, 358-361.

Prieta, J., A. M. Domenech, J. F. Fernandez-Grayzabal, M. D. Collins, U. M. Rodrigues, D. Jones, A. Rodrigues, L. Dominguez, 1993 Lactococcicosis de la Trucha Arco Iris (*Oncorhynchus mykiss*). Med. Vet. Vol 10, (6) 367-373.

Ravelo, C., B. Magarinos, J. L. Romalde, A. E. Toranzo (2001) Conventional versus miniaturized systems for the phenotypic characterization of *Lactococcus garvieae* strains. Bull. Eur. Ass. Fish Pathol. 21(4) 136-144.

Salati, F., P. Tassi, P. Bronzi, 1996. Isolation of *Enterococcus* like Bacterium from Diseased Adriatic Sturgeon *Acipenser naccarii*, Farmed Italy. Bull. Eur. Ass. Fish Pathol. 16 (3) 96-99.

Sneath, P. H. A., N. S. Mair, M. E. Sharpe, J. G. Holt, 1986. Bergey's Manual of Systematic Bacteriology, Vol 2.Williams and Wilkins, Baltimore.

Teixeira, L. M., V. L. C. Merquior, M. C. E. Vianni, M. G. S. Carvalho, S. E. L. Fracalanzza, A. G. Steigerwalt, D. J. Brenner, R. R. Facklam, 1996. Phenotypic and genotypic characterization of atypical *Lactococcus garvieae* strains isolated from water buffalos with subclinical mastitis and confirmation of *L. garvieae* as a senior subjective synonym of *Enterococcus seriolicida*. Int. J. Syst. Bacteriol. 46: 664–668.

Toranzo, A. E., S. Devesa, P. Heinen, A. Riaza, S. Nun˜ez, J. L. Barja, 1994. Streptococcosis in cultured turbot caused by an enterococcus-like bacterium. Bull. Eur. Assn. Fish Pathol. 14: 19–23.

Toranzo, A. E., A. M. Baya, B. S. Roberson, J. L. Barja, D. J. Grimes, F. M. Hetrick (1987). Specificity of slide agglutination test for detecting bacterial fish pathogens. Aquaculture 61,2,81-97.

Toranzo, A. E., S. Devesa, P. Heinen, A. Riaza, S. Nunez, J. L. Barja, 1994. Streptococcosis in Cultured Turbot Caused by an *Enterococcus*-like Bacterium. Bull. Eur. Ass. Fish. Pathol. 14 (1) 19-23.

Vela, A. I., C. Vazquez, A. Gibello, M. M. Blanco, M. A Moreno, P. Liebana, C. Albendea, B. Alcala, A. Mendez, L. Dominguez, J. F. Fernandez-Garayzabal (2000). Phenotypic and Genetic Characterization of *Lactococcus garvieae* Isolated in Spain from Lactococcosis Outbreaks and Comparison with Isolates of Other Countries and Sources. J. Clin. Microb. 38, 10:3791-3795.

Walbanks, S., A. J. Martinez-Murcia, J. L. Fryer, B. A. Phillips, M. D. Collins, 1990.16 S RNA Sequence Determination for Members of Genus *Carnobacterium* and Related Lactic Acid Bacteria and Description of *Vagococcus salmoninarum* sp. nov. Int. J. Syst. Bacteriol. 40, 224-230.

Williams, A. M., J. L. Fryer, M. D. Collins, 1990. *Lactococcus piscium* sp. nov. A new *Lactococcus* species from Salmonid Fish. FEMS Microbiol. Lett. 68, 109-114.