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Lactococcus garvieae strains isolated from rainbow trout and yellowtail in Australia, South Africa and Japan differentiated by repetitive sequence markers

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Abstract

Lactococcus garvieae is an emerging important epizootiological pathogen. A total of 25 *Lactococcus garvieae* isolates from diseased rainbow trout (*Oncorhynchus mykiss*) and yellowtail (*Seriola quinqueradiata*) from Japan, South Africa and Australia (Tasmania and Victoria) and type strains were analysed by repetitive sequence based PCR targeting repetitive extragenic palindromes (REP) and BOX interspersed DNA sequences. Both primer sets generated five different banding profiles with isolates from South Africa, Tasmania and Victoria sharing common profiles. Japanese isolates could easily be distinguished from all other isolates on the basis of banding profile and BOX primers enabled differentiation of capsular types for these strains. The type strain *L. garvieae* NCFB 2157 produced a unique profile significantly different from other strains with both oligonucleotide primer sets. REP-PCR and BOX-PCR offer a rapid, cost effective and straightforward means for strain differentiation of this important fish and emerging zoonotic pathogen.

Introduction

Members of the genus *Lactococcus* have previously been considered unimportant clinical isolates from animals and humans, however evidence has emerged which proves some members of this taxon are important pathogens of a number of animal species including humans (Teixera et al., 1996). *Lactococcus garvieae* originally isolated from cases of bovine mastitis is now a well recognised pathogen of cultured fish species in Japan, Australia, Italy, Spain and Israel (Austin & Austin, 1999, Vela et al., 2000) and is an emerging zoonotic pathogen (Zlotkin et al., 1998). Genomic analysis has established that *Enterococcus seriolicida* is a junior synonym of *L. garvieae* (Eldar et al., 1996). Differentiation of pathogenic strains of *L. garvieae* from non-pathogenic species of the *Lactococcus* and *Enterococcus* genera is difficult by conventional biochemical testing due to the limited phenotypic variation displayed by this group of organisms (Elliott & Facklam, 1996, Eldar et al., 1999). Gene probes have been developed which enable species identification of both this organism (Aoki et al., 2000) and other bacterial fish pathogens (Carson, 1998) but are not able to differentiate strains, essential for epizootiological investigations. The use of PCR based techniques for amplification of DNA flanked by targeted repetitive sequences, enables rapid strain delineation for

epidemiological purposes. Sequences such as Repetitive Extragenic Palidromic (REP), Enterobacterial Repetitive Intergenic Consensus (ERIC) (Versalovic et al., 1991) and BOX elements (Koeuth et al., 1995) have been used with results comparable to pulsed field gel electrophoresis (PFGE) (Wong & Lin, 2001) which is considered to be the most reliable and reproducible method for bacterial strain identification (Olive & Bean, 1999). In this study *L. garvieae* isolates from geographically distinct locations and piscine host species were analysed by REP-PCR and BOX-PCR to determine the usefulness of these methods for epizootiological purposes.

Materials and Method

Source of isolates

Twenty five strains of *Lactococcus garvieae*, including two type strains, three isolates from South Africa, three from Japan representing two different capsular phenotypes KG- and KG+ (Yoshida et al., 1996) and 17 from Australia were used in this study (Table 1). Australian isolates comprised two isolates obtained from diseased fish in Victoria and 15 isolates from fish in Tasmania. Isolates from Tasmania were selected from a collection obtained from disease outbreaks in rainbow trout in geographically distinct locations within the state over a four year period.

PCR

Isolates were grown in aerobic conditions overnight on Blood Agar Base No. 2 (Oxoid CM271) supplemented with 7% v/v defibrinated sheep's blood (SBA) at 25°C. Genomic DNA was extracted from isolates using a DNeasy Tissue Kit (Qiagen) in accordance with the manufacturers instructions for Gram positive bacteria. The concentration of DNA was determined spectophotometrically at 260 nm (GeneQuant, Amersham) and diluted to 50 ng μl^{-1} for amplification. For REP PCR the primers REP1R-Dt (5'-IIIICGICGICATCIGGC-3') and REP2-Dt (5'-ICGICTTATCIGGCCTAC-3') (Versalovic et al., 1991) and for BOX PCR the BOXA2R primer (5'-ACGTGGTTTGAAGAGATTTTCG-3' (Koeuth et al., 1995) were used. PCR amplification was performed using Platinum Taq PCRx[J.1] DNA polymerase (Invitrogen). Amplifications were performed in a reaction volume of 25 µL containing $MgCl_2$ (2.0 mM REP, 1.5 mM BOX), 0.625 mM each dNTP, 50 pM primers (each and opposing REP) or 100 pM primer (BOX) and 0.625 U Taq. Amplification conditions used were initial denaturation at 94°C for 3 min, annealing at 42°C for 1 min REP or at 40°C for 1 min BOX, extension at 72°C for 8 min and cycle denaturation at 94°C for 30 sec. A negative control was included with each PCR.

Analysis of amplicons

Amplicons were separated by electrophoresis at 70 V for 60 min (BOX) or 90 min (REP) using 1.0% agarose (Promega) in Tris acetate EDTA buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) then stained with ethidium bromide (0.5 µg mL^{-1}) for 40 min and destained in water for 40 min prior to ultraviolet transillumination. Ecor R1 digested SPP-1 bacteriophage DNA (GeneWorks) was used as size markers. Band positions were recorded by Polaroid image capture.

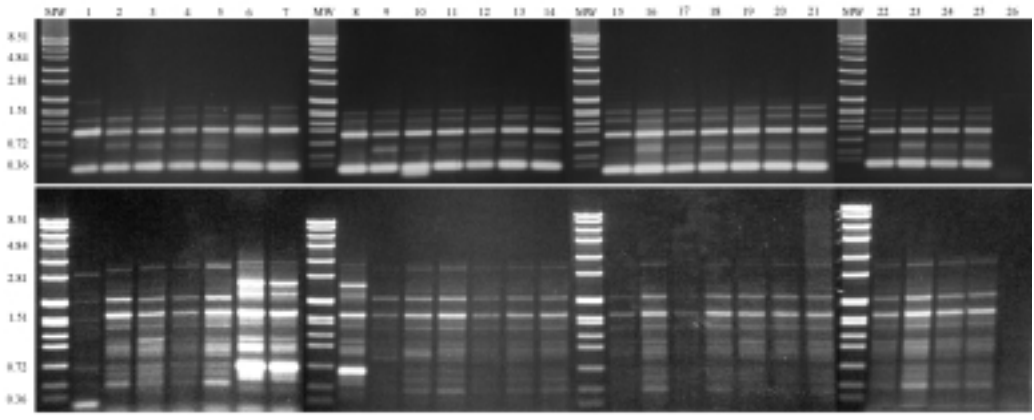

Figure 1. Agarose gels of PCR products obtained from *Lactococcus garvieae* isolates in this study generated from whole cell DNA using REP (top gel) and BOX (bottom gel) primers. Type strains (lane 1 & 2) and fish isolates obtained from South Africa (lanes 3-5), Japan (lanes 6-8), and Australia (lanes 9-25). Lane numbers correspond to Table 1. MW; molecular weight markers (Ecor-R1 digested SPP-1 bacteriophage, Gene Works).

Results

PCR analysis using both REP and BOX oligonucleotides generated five discriminate banding patterns for each primer set, for the 25 strains of *Lactococcus garvieae* examined in this study, as illustrated in Fig. 1. *L. garvieae* NCFB 2157 (lane 1) produced unique banding profiles with both BOX and REP PCR designated BOX-I and REP-I respectively. The most common BOX profile, designated BOX-II, was generated from 20 isolates comprising all strains isolated in Tasmania, one from Victoria (V83/3505-1), all the South African isolates and *L. garvieae* ATCC 49156; the Australian and South African strains all came from rainbow while ATCC 49156 was isolated from yellowtail in Japan. The remaining strains of *L. garvieae* from Japan were all isolated from yellowtail have a significantly different banding profile; BOX-III (phenotype KG+, lane 6), lacking fragments at ~1.53 and 0.69 Kb, and BOX-IV (phenotype KG-, lane 7 and 8) lacking a band at ~ 0.69 Kb. BOX-V has the same banding pattern as BOX-II but contains an additional band at ~0.31 Kb and was represented by one isolate from Victoria (V87/1039-1 lane 10).

The most frequent REP profile, designated REP-II, was generated by *Lactococcus garvieae* ATCC 49156, all isolates from South Africa, one strain from Victoria (V87/1039-1) and 14 strains from Tasmania. One Tasmanian strain (88/1400-1B, lane 15) differed in profile from REP-II by lacking a band at ~0.82 Kb and is designated REP-V, whilst one strain from Victoria (V83/3505-1, lane 9) varied from REP-II by lacking bands at ~0.67, 0.56 and 0.45 Kb and is designated REP IV. The remaining *L. garvieae* isolates from Japan were designated as profile REP-III with appreciable differences in profile to REP-II, however no discrimination between capsular phenotypes KG- and KG+ (lanes 6-8) can be made with this primer set.

Differences in banding intensity for REP profiles at matching positions of ~0.82, 0.56 and 0.45 Kb for some isolates (lanes 9 and 15) cor-

Lane number	Collection/Strain No.	Origin	Host*	REP Profile	BOX Profile
1	NCFB 2157^T	United Kingdom	bovine	I	I
2	ATCC 49156^T	Japan	yellowtail	II	II
3	SA2 7876	South Africa	rainbow trout	II	II
4	SA1 7845	South Africa	rainbow trout	II	II
5	43/87	South Africa	rainbow trout	II	II
6	KG+HP9501-1	Japan	yellow tail	III	III
7	KG-MZS9501-2	Japan	yellowtail	III	IV
8	KG-HP9502-2	Japan	yellowtail	III	IV
9	V83/3505-1	Australia (Victoria)	rainbow trout	IV	II
10	V87/1039-1	Australia (Victoria)	rainbow trout	II	V
11	88/595	Australia (Tasmania)	rainbow trout	II	II
12	88/1209	Australia (Tasmania)	rainbow trout	II	II
13	88/3873-1	Australia (Tasmania)	rainbow trout	II	II
14	86/260	Australia (Tasmania)	rainbow trout	II	II
15	88/1400-1B	Australia (Tasmania)	rainbow trout	V	II
16	89/1592-2	Australia (Tasmania)	rainbow trout	II	II
17	89/1593	Australia (Tasmania)	rainbow trout	II	II
18	88/3998-69	Australia (Tasmania)	rainbow trout	II	II
19	88/3998-79	Australia (Tasmania)	rainbow trout	II	II
20	88/1317	Australia (Tasmania)	rainbow trout	II	II
21	88/1508	Australia (Tasmania)	rainbow trout	II	II
22	88/465	Australia (Tasmania)	rainbow trout	II	II
23	90/3874-2bl	Australia (Tasmania)	rainbow trout	II	II
24	90/723-113	Australia (Tasmania)	rainbow trout	II	II
25	88/3425-6	Australia (Tasmania)	rainbow trout	II	II
26	PCR negative control				

Table 1. Origins, REP and BOX profile designations and lane identification of *Lactococcus garvieae* strains used in this study. Lane number refers to Fig. 1. ^{T} Type strain; * rainbow trout, *Oncorhynchus mykiss*; yellowtail, *Seriola quinqueradiata*

responding to profiles REP-IV and V respectively made interpretation somewhat difficult, however repeated analysis with these isolates did not materially improve banding resolution.

Discussion

Genomic analysis to determine bacterial strain relatedness has become the preferred means of investigation for epidemiological purposes particularly when the phenotype of the isolates of interest does not provide a suitable level of discrimination. A number of tech-

niques have been developed with PFGE being considered to have the highest power of discrimination for strain identification (Olive & Bean, 1999). However PFGE has the disadvantage of requiring considerable technical expertise, expensive equipment and is less timely than PCR based typing methods such as random amplified polymorphic DNA (RAPD) assays. A derivative of RAPD analysis, using PCR based upon repetitive elements within the bacterial genome, REP-PCR was initially described as a tool for strain identification by Versalovic et al., (1991). Identification of additional conserved sequences within bacterial genomes (Koeuth et al., 1995) expanded the range of primers targeting repetitive sequences with potential for PCR based strain identification. REP-PCR has now become a widely used tool for epidemiological investigations of a range of human, veterinary and plant bacterial pathogens.

Lactococcus garvieae isolates examined in this study could readily be assigned to one of five groups using either REP or BOX primers. Isolates from Tasmania, Victoria and South Africa formed a relatively homologous group with REP profiles and is in agreement with previous work with these strains by Carson et al., (1993) who demonstrated a high degree of similarity by phenology, serology and expressed proteins. Variation in banding intensity and band absence compared with the most common REP profile (REP-II) for two of these strains has resulted in two additional REP profiles (REP-IV and V) each comprised of only one strain and may be an artefact rather than truly representative of strain difference.

Japanese isolates representative of different capsular phenotypes (KG- and KG+) are significantly different from all other isolates analysed and are grouped into separate REP and BOX profiles. REP profiles cannot distinguish capsular phenotypes however BOX profiles vary by the absence of a band at 1.53 Kb for the KG+ strain. KG- capsular phenotypes have been reported to possess a specific envelope capsule and is more virulent in yellowtail than the KG+ phenotype (Alim et al., 1996; Yoshida et al., 1997). Further investigation is required to confirm the ability of BOX-PCR to adequately differentiate KG-and KG+ capsular phenotypes of *Lactococcus garvieae*.

Interestingly the ATCC 49156 strain, isolated from diseased yellowtail in Japan, exhibited REP and BOX profiles identical to Tasmanian, Victorian and South African isolates. However the type strain NCFB 2157, originally isolated from a mastitic bovine udder was determined to have a unique REP and BOX profile that differentiated this strain from the fish isolates. The distinctiveness of the type strain was found by Eldar et al., (1999) using RFLP ribotyping to determine strain variation of *Lactococcus garvieae* isolates from fish.

In conclusion, we have demonstrated that REP-PCR and BOX-PCR offer rapid and reliable means for strain differentiation of *Lactococcus garvieae* isolated from piscine samples. Variability in REP and BOX profiles may be attributed to geographical location of strain isolation and possibly capsular phenotypes. REP and BOX PCR techniques enables rapid means for discrimination of strain relatedness for this pathogen.

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