EVALUATION OF BIOLOG'S MICROLOG SYSTEM FOR THE IDENTIFICATION OF GRAM POSITIVE COCCI PATHOGENIC FOR FISH

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Introduction

Many Gram-positive cocci (GPC) have been described as being responsible for disease in a range of freshwater or marine fish species. The list of GPC pathogenic for fish now includes at least seven different species: *Streptococcus iniae*, *S. parauberis*, *S. agalactiae*, *Lactococcus piscium*, *L. garvieae* and *Vagococcus salmoninarum*.

The identification of such pathogens using conventional bacteriological techniques has always been considered to be difficult and has often led to misdiagnosis. Such methods depend upon the characterisation of the phenotypic and biochemical patterns of each isolate followed by a comparison of these profiles with published references. Biochemical variations amongst strains isolated from different fish species or geographical areas, methodological variations between laboratories and, to some extent, a lack of accurate data have made the use of published profiles and miniaturised biochemical tests such as the Rapid ID 32 Strep system (Biomérieux, France) unreliable for accurate identification of fish pathogenic GPC. For these reasons, many workers have turned to the development and validation of molecular biological tools such as 16s rRNA sequencing or the polymerase chain reaction for the identification of such pathogens. Many of these tools have now been developed and are being used to identify these organisms accurately. These techniques are however expensive and time consuming and, although powerful, are not available in all fish diagnostic laboratories.

We have investigated the use of Biolog's Microlog System (Biolog, USA) to identify and characterise some of the Gram-positive cocci listed above together with other GPC that may be markers of environmental contamination. Unlike other secondary tests, the Microlog System can recognise over $4x10^{28}$ possible metabolic patterns by testing the ability of test bacteria to use as many as 95 different carbon sources. The test results in a pattern or fingerprint, which is claimed to be unique to the test bacterium and readable visually. The fingerprint data having been fed into Microlog software, an identification may be possible within 4 to 6 hours.

A comparison of the results obtained for the identification of a number of pathogenic and non-pathogenic GPC using both the Rapid ID 32 Strep and Microlog systems is presented below.

Material and methods

Bacterial strains and culture conditions

The twenty-three *Lactococcus garvieae* isolates tested in this study, their host, year of isolation and geographical origin are shown in Table 1. A number of reference isolates were also included in the study for comparative purposes. Details of these isolates are given in Table 2.

The identity of all isolates was confirmed prior to the start of the study by 16s rRNA sequencing and comparison of the obtained sequences to the bacterial 16s sequences submitted to GenBank and EMBL databases using the BLAST search program available at the UK HGMP Resource Centre, Hinxton.

RAPID ID 32 STREP test

The RAPID ID 32 STREP is a standardised system for the identification of streptococci and related organisms. The system uses 32 miniaturised enzymatic tests each containing a dehydrated test substrate. When inoculated with a bacterial suspension the enzymatic substrates are rehydrated and the metabolic end products produced during incubation detected. Identification is achieved using the Biomérieux identification software and/or by comparison to Ravelo *et al.* (2001) profiles.

In order to achieve an accurate identification of the test isolates two RAPID ID 32 STREP tests were carried out for each isolate. The first following the procedure outlined in the instruction manual accompanying the test and the second using a modified method recommended by Ravelo *et al.* (2001) for repetitive and accurate identification of *Lactococcus garvieae* isolates.

The conventional method involved first growing the bacteria on Columbia sheep blood agar (Sigma, USA) supplemented with sheep blood (Oxoid Ltd, UK) for 18-24 hours at 37°C. A suspension of the bacterium was then made in sterile distilled water to a McFarland standard of 4. Each test well was then inoculated with 55µl of the suspension. A small amount was also added to a Tryptone soya agar (TSA, Oxoid) plate to act as a purity check. Both were then incubated at 37°C, and the results recorded after 4-4.5 hours.

Picture 1: Inoculation of an API RAPID ID 32 Strep test

To perform the modified method, the bacteria was again grown on Columbia sheep blood agar, but was incubated at 25°C for 24-48h. The optical density of the bacterial suspension was spectrophotometrically adjusted (0.8 at 580nm) and the incubation temperature for the test strip was lower at 25°C. Results were recorded after 4-4.5 hours.

Biolog System

The GP2 Microplate is another standardised system for the identification and characterisation of a broad range of Gram positive bacteria by their ability to utilise or oxidise compounds from a pre-selected panel of carbon sources. The system uses 95 miniaturised biochemical tests each containing a different dehydrated carbon source and a redox dye. When inoculated with a bacterial suspension (in a gelling' inoculating fluid) the tests are rehydrated and during incubation there is a burst of respiration in the wells containing chemicals that can be oxidised. The bacterial cells then reduce the dye forming a purple colour and yielding a characteristic pattern of purple wells. Identification is achieved using Biolog's MicroLog 1 software.

To characterise the test isolates using the Biolog system, the bacteria were subbed twice onto BUG agar with 5% sheep blood and incubated under optimum growing conditions (previous work indicated a temperature of 30°C for 20±2h). After the second incubation period, three drops of the anti-capsular agent sodium thioglycolate were added to a tube of sterile Gram positive inoculating fluid and a suspension of the bacterium prepared to a 20% (±2%) transmittance level. Each test well was then inoculated with 150µl of the bacterial suspension, a small amount also added to a TSA plate to act as a purity check. Both were then incubated at 30°C, and the results recorded after 4-6 and 16-24 hours.

Picture 2: Characteristic pattern obtained on a Biolog GP2 plate inoculated with Lactococcus garvieae (4-6h incubation)

Table 1: Origin of the Lactococcus garvieae isolates used in this study

Reference	Origin	Year of isolation
01/115 – ATCC 49156 – YT-3	Yellowtail, Japan	1974
02/087 – NCIMB 702927	Fish	N/A
00/021	Rainbow trout, UK	2000
00/022	Rainbow trout, UK	2000
00/024	Rainbow trout, UK	2000
00/025	Rainbow trout, UK	2000
01/141	Rainbow trout, UK	2001
01/144	Rainbow trout, UK	2001
01/145	Rainbow trout, UK	2001
01/150	Rainbow trout, UK	2001
PP 60.1	Rainbow trout, Spain	1997
02/105	Rainbow trout, Spain	2000
B(2001)	Rainbow trout, Turkey	2001
657/1	Rainbow trout, Italy	1999
657/2	Rainbow trout, Italy	1999
2913	Rainbow trout, Italy	1999
325/I/2001	Rainbow trout, Italy	2001
36/F/2002	Rainbow trout, Italy	2002
74/F/2002	Rainbow trout, Italy	2002
03/063	Rainbow trout, Europe	–
03/064	Yellowtail, Japan	–
29.99	Rainbow trout, France	1999
IZP 116.78	Rainbow trout, France	–

Table 2: Origin of the other Gram positive bacterial reference isolates used in this study

Reference Isolates	Bacterial isolate	Origin[1]
01/162 – NCIMB 8662	*Lactococcus lactis cremoris*	New Zealand
01/163 – NCIMB 6681	*Lactococcus lactis lactis*	NS
02/123 – NCIMB 13196	*Lactococcus piscium*	Rainbow trout, USA
01/189 – ATCC 29178	*Streptococcus iniae*	Amazon freshwater dolphin
01/165 – NCIMB 8778	*Streptococcus agalactiae*	NS
01/164 – NCIMB 12756	*Enterococcus faecalis*	NS
02/031 – NCIMB 2264	*Carnobacterium piscicola*	Cutthroat trout, USA
03/121 – NCIMB 13133	*Vagococcus salmoninarum*	Rainbow trout, USA
03/122 – NCIMB 702779	*Vagococcus salmoninarum*	Rainbow trout, USA

Note1: NS = Not Specified

Results

Table 3 presents the results of our API RAPID ID 32 STREP tests using both the conventional and the modified methodologies. As shown, none of the test methods performed on our 23 *Lactococcus garvieae* isolates gave a reliable identification. These methods also failed to correctly and reliably identify other GPC, which may be potential fish pathogens or environmental contaminants.

Tables 4 and 5 present the results of our Biolog tests. Visual microplate reading after only 4-6h incubation proved difficult. After such a short incubation period, 79% of the *Lactococcus garvieae* isolates and 55% of the other GPC were however correctly identified. The rest of the isolates were not identified. After 16-24h incubation, 22/23 of the tested *Lactococcus garvieae* isolates were correctly identified. The test also allowed us to correctly identify most of the other GPC. *Lactococcus piscium* was unable to grow on BUG agar making its diagnosis and identification impossible with this technique.

Picture 3: Diseased Rainbow trout showing typical clinical signs of a Lactococcus garvieae infection

Table 3: Identification of Lactococcus garvieae and other fish pathogenic and non-pathogenic Gram positive bacteria using API RAPID ID 32 STREP. Number of correctly identified isolates using either the API database or Lactococcus garvieae profiles given by Ravelo et al. (2001)

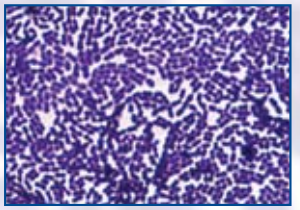
Isolate details	Number of correctly identified isolates		
	Conventional RAPID ID 32 STREP Test. Identification using the API database (V 2.0)	Modified RAPID ID 32 STREP Test as per Ravelo *et al.* (2001)	
		Identification using the API database (V2.0)	Identification using Ravelo *et al.* (2001) (2001) profiles
Lactococcus garvieae Field isolates	9 out of 21	13/21	0/21
Lactococcus garvieae Reference isolates	1/2	1/2	0/2
Lactococcus lactis cremoris	1/1	1/1	N/A
Lactococcus lactis lactis	0/1	1/1	N/A
Lactococcus piscium	0/1	0/1	N/A
Streptococcus iniae	0/1	0/1	N/A
Streptococcus agalactiae	0/1	0/1	N/A
Enterococcus faecalis	0/1	1/1	N/A
Carnobacterium piscicola	0/1	0/1	N/A
Vagococcus salmoninarum	No growth	1/2	N/A

Table 4: Biolog results of the 23 Lactococcus garvieae isolates tested in this study. GP2 microplates read after the recommended incubation periods of 4-6 and 16-24h

Isolate details	Result following 4-6h incubation				Result following 16-24h incubation			
	First species ID	Prob (%)	Sim	Dist	First species ID	Prob (%)	Sim	Dist
ATCC 49156	**No ID**				*Lactococcus garvieae*	**74**	0.625	2.4
NCIMB 702927	**No ID**				*Lactococcus garvieae*	**100**	0.880	1.79
00/021	*Lactococcus garvieae*	87	0.795	1.26	*Lactococcus garvieae*	**100**	0.894	1.57
00/022	*Lactococcus garvieae*	89	0.089	1.26	*Lactococcus garvieae*	**100**	0.585	6.46
00/024	*Lactococcus garvieae*	99	0.893	1.5	*Lactococcus garvieae*	**100**	0.904	1.43
00/025	*Lactococcus garvieae*	99	0.919	1	*Lactococcus garvieae*	**100**	0.814	2.79
01/141	*Lactococcus garvieae*	87	0.794	1.26	*Lactococcus garvieae*	**100**	0.818	2.72
01/144	*Lactococcus garvieae*	88	0.809	1.26	*Lactococcus garvieae*	**100**	0.932	1
01/145	*Lactococcus garvieae*	94	0.826	1.78	*Lactococcus garvieae*	**100**	0.897	1.53
01/150	*Lactococcus garvieae*	100	0.836	2.45	*Lactococcus garvieae*	**100**	0.903	1.43
PP 60.1	*Lactococcus garvieae*	90	0.76	2.26	*Lactococcus garvieae*	**100**	0.75	3.76
02/105	**No ID**				*Lactococcus garvieae*	**100**	0.972	0.41
B(2001)	*Lactocccus garvieae*	99	0.843	2.26	*Lactococcus garvieae*	**100**	0.858	2.11
657/1	*Lactococcus garvieae*	98	0.827	2.34	*Lactococcus garvieae*	**100**	0.901	1.47
657/2	*Lactococcus garvieae*	100	0.898	1.48	*Lactococcus garvieae*	**100**	0.901	1.47
2913	*Lactococcus garvieae*	97	0.792	2.7	*Lactococcus garvieae*	**100**	0.859	2.1
325/I/2001	*Lactococcus garvieae*	99	0.822	2.57	*Lactococcus garvieae*	**100**	0.895	1.57
36/F/2002	**No ID**				*Lactococcus garvieae*	**100**	0.923	1.14
74/F/2002	*Lactococcus garvieae*	95	0.796	2.45	*Lactococcus garvieae*	**100**	0.839	2.41
03/063	**Not read**				*Lactococcus garvieae*	**99**	0.800	3.00
03/064	**Not read**				*Lactococcus garvieae*	**99**	0.572	6.70
29.99	*Lactococcus garvieae*	100	0.93	1.04	**No ID**			
IZP 116.78	**No ID**				*Lactococcus garvieae*	**100**	0.73	4.15

Table 5: Biolog results of the 9 other Gram positive fish and non-fish bacterial pathogens tested in this study. GP2 microplates read after the recommended incubation periods of 4-6 and 16-24h

Isolate details	Result following 4-6h incubation				Result following 16-24h incubation			
	First species ID	Prob (%)	Sim	Dist	First species ID	Prob (%)	Sim	Dist
NCIMB 8662 *Lactococcus lactis cremoris*	*L. lactis cremoris*	100	0.84	2.39	*L. lactis cremoris*	100	0.909	1.36
NCIMB 6681 *Lactococcus lactis lactis*	*L. lactis lactis*	100	0.851	2.23	*L. lactis lactis*	100	0.583	6.5
NCIMB 13196 *Lactococcus piscium*	no growth on BUG agar							
ATCC 29178 *Streptococcus iniae*	**No ID**				*S. iniae*	100	0.87	2.00
NCIMB 8778 *Streptococcus agalactiae*	**No ID**				**No ID**			
NCIMB 12756 *Enterococcus faecalis*	**No ID**				*E. faecalis*	98	0.886	1.46
NCIMB 2264 *Carnobacterium piscicola*	*C. piscicola*	100	0.929	1	*C. piscicola*	100	0.719	4.27
NCIMB 13133 *Vagococcus salmoninarum*	*V. salmoninarum*	100	1.00	0.00	*V. salmoninarum*	100	0.90	1.43
NCIMB 702779 *Vagococcus salmoninarum*	**No ID**				***Misidentified*** *as V. fluvialis*	98	0.73	3.97



Picture 4: Gram stained Lactococcus garvieae

Conclusion

The API RAPID ID 32 STREP modified method suggested by Ravelo *et al.* (2001) allowed us to get better results for the identification of *Lactococcus garvieae* than when using the conventional Biomérieux method. The technique was however not reliable and did not allow an accurate identification of all GPC tested in this study.

The Biolog method on the contrary accurately identified 22/23 *Lactococcus garvieae* isolates and all but three of the other GPC included in this study when the full 16-24h incubation period was allowed to take place. Good IDs were already obtained after only 4-6h incubation.

In view of these results we would strongly recommend the use of Biolog's microlog system for the identification of *Lactococcus garvieae*. In terms of cost, the Biolog system, although dearer in terms of consumables, is also cheaper to use for such purpose than the API system when staff time and cost are taken into consideration.

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References

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