Sensitive Diagnostic tools to screen for Gaffkemia infection in European lobsters (*Homarus gammarus*)

A Scientific Report by

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Introduction to the project

Tarbert Shellfish Ltd. is a company that processes live shellfish. It uses high-density holding facilities to accumulate stock prior to transport and export. However, any infections brought into the communal hold from different locations around Scotland can spread through the stock, resulting in substantial financial losses, which threatens their profitability, and in turn the livelihood of the Scottish creel fishermen supplying the company.

A particular problem has recently arisen on more than one occasion that has been thought to be due to the highly contagious marine bacterium *Aerococcus viridans homari*, and an urgent need exists for a sensitive diagnostic tool to screen incoming stock for this infection (commonly called Gaffkemia). The company does not have the scientific knowledge or resources to carry out this work, and therefore wishes to enter into a partnership with researchers at the Langoustine Lab at the University of Glasgow, led by Professor Douglas Neil, which has a known track record within the crustacean seafood industry.

Objectives

Diagnostic identification of heavy infections of *Aerococcus* are currently based on microscopical examination of blood smears for the characteristic tetrad formations of the bacterial cocci. This project will develop a more sensitive standard protocol for the diagnosis of subclinical infections of *Aerococcus viridens* var. *homari*, involving both a screening process and confirmatory techniques based on the culture, isolation and biochemical characterization of the pathogen. A critical assessment of the effectiveness of adapting commercial assay kits as diagnostic tools will also be conducted. With this significant improvement in detection sensitivity the company will be able to screen for infection on-site, and also to assess the success of disinfection measures.

Materials and Methods

Media preparation used in this project

Nutrient agar (NA):

Lamb-Lemco beef extract – 0.75 g

Yeast extract – 1.5 g

Peptone – 3.75 g

NaCl – 3.75 g

Agar – 11.25 g

Adjust volume to 750 ml with distilled water and proceed to autoclave media.

Nutrient Agar Broth (NB):

Lab-Lemco – 0.4 g

Yeast extract – 0.8 g

Peptone – 2 g

NaCl- 2 g

Adjust volume to 400 ml with distilled water and proceed to autoclave media.

Tryptone Soya Agar (TSA):

TSA powder (CM0131, from Oxoid) - 16 g

Adjust volume to 400 ml with distilled water and proceed to autoclave media.

Tryptone Soya Broth (TSB):

TSB powder (CM0129, from Oxoid) – 12 g

Adjust volume to 400 ml with distilled water and proceed to autoclave media.

Reference strain of Aerococcus viridans

Aerococcus viridians reference strain NCIMB 1119 (batch reference 16021983) was purchased from NCIMB Limited (Aberdeen, Scotland). Lyophilised strain resuscitation was performed following manufacturer's instructions. In summary, lyophilised bacteria were re-suspended in 200 µl of TSB media and drops of this suspension were plated in

NA and TSA plates (5 plates for each medium). Plates were incubated at 28 ºC for 24 h. After this time single colonies from TSA and NA plates were re-cultured in fresh TSA and NA plates. Colonies from this second culture were re-suspended in TSB and NB culture media containing glycerol (15%) and kept at -80 ºC until needed.

Biochemical test StrepQuick

StrepQuick was obtained from Hardy Diagnostis, California, USA. The catalogue number was Z122. The test is intended to aid in the identification of gram-positive, catalase-negative cocci based on the reactions pyroglutamate aminopeptidase (PYR), leucine aminopeptidase (LAP) and esculin hydrolysis (ESC) activity. This test is to be used only with cultures of isolated organisms. Test circles are slightly moisten by addid a single drop of distilled water. Using a sterile plastic loop, we pick 2-3 isolated 18-24 h colonies and rub them into a small area of the PYR reaction circle. This step is repeated for the LAP and ESC test circles. After the test organism has been inoculated onto the test circles we allow it to react for 10 min. After 10 min we add one drop of the chromogenic developer to the PYR and LAP circles and immediately after addition of this component we observe for the development of a bright pick or cherry red colour in the PYR and LAP circles. Also observe for a light grey to grey colour to form in the ESC circle. Interpretation of results is as follows:

Test	Positive Reaction	Negative reaction
PYR	Cherry red or bright pink colour	Orange, yellow or salmon colour or no change
LAP	Cherry red or bright pink colour	No colour development
ESC	Light grey to grey colour	Any colour other than grey

Expected results for *A. viridans* would be as follows:

PYR: Variable results

LAP: Negative, no colour change

ESC: Variable results

Biochemical test Api20Strep

The Api20 STREP strip consist of 20 microtubes containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars. This enzymatic tests have to be inoculated with a dense suspension of organisms coming from a pure culture, which is used to rehydrate the enzymatic substrates. The metabolic end products produced during the incubation period are either revealed through spontaneous colored reactions or by the addition of reagents. The reactions are read according to the Interpretation Reactions given by the manufacturer into positive or negative results. The compiled results is transformed into a code numeric profile and the identification of the organisms is done using the software apiweb^{TM}. The principle of the code profile is to condense the binary information (+,-) into a numeric profile. Test on the strip are separated into groups of 3 and to each reaction a value equal to 1, 2 or 4 is attributed depending on the position of the test in tis group, 1sr, 2nd or 3rd respectively. The addition of the 3 retained values (0 for negative reactions) gives a figure between 0 and 7 for each group. The resulting profile is matched to a database. Based on the accuracy of the identification the following identifications are possible:

- Excellent: % i.d. ≥ 99.9 % and T ≥ 0.75
- Very good: % i.d. ≥ 99.0 % and T ≥ 0.50
- Good: % i.d. ≥ 90.0 % and T ≥ 0.25
- Acceptable: % i.d. ≥ 80.0 % and T ≥ 0.0
- Unacceptable: when the profile is very far from the taxa of the database, all gross frequencies being less than the threshold values.

Isolation of the bacterial micro-flora DNA for amplification and sequencing

Single bacterial colonies were picked randomly with sterile scrapers from TSA or PEA plates. A fraction of the bacteria colony was dissolved in 100 µl of sterile-filtered distilled H2O. The samples were heated at 100 ºC for 10 min and subseuqnently cooled on ice. This step denatures all proteins within the sample and thereby releases genomic DNA from the cells. Samples were either immediately processed for polychain reaction (PCR) or frozen at -20 ºC until needed.

Amplification and sequencing of DNA

A 1 µl volume of crude DNA preparation was used per PCR reaction. The partial DNA fragments of bacterial 16S rRNA genes were amplified by PCR using a forward primer 27F (equivalent to positions 8 to 27 in *Escherichia coli* 16S rRNA) and a reverse primer 1100R (equivalent to positions 1114 to 1110 in *Escherichia coli* 16S rRNA) (Table 1). The PCR mixture (50 µl) was set up with 10 µl of 5x PCR-buffer containing 10 mM $MgCl_2$, 20 mM dNTPs (1 µl), 0.25 µl Taq-polymerase, 1.5 µl of each primer (stocks at 10 µM), 34.75 µl of distilled and sterile H2O and 1 µl of DNA template. The PCR conditions were: 94 ºC for 5 min, followed by 35 cycles of 94 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 2 min with a subsequent extension cycle at 72 ºC for 10 min. A 10 µl aliquot of the amplified product was visualized on a 1.0 % agarose gel stained with containing 10 µg/ml (w/v) of ethidium bromide. The remaining 40 µl volume of the PCR reaction was purified using a QIAqucik PCR purification kit (QUIAGEN) following the supplied protocol and send for sequencing.

Table 11. Primers used for 16S-rDNA amplification and sequencing.

Primer name	Sequence
27-F	5'-AGA GTT TGA TCM TGG CTC AG-3'
685-R	5'-TCT ACG CAT TTC ACY GCT AC-3'
1100-R	5'-GGG TTG CGC TCG TTG-3'

Results

Sampling of lobsters in Tarbert Fish farm

MARCH SAMPLING – On the 16/03/2010 4 lobsters with no signs of disease were taken from Tarbert fish farm to the laboratory in Glasgow University by ice where they were sampled. Furthermore, 2 samples of sediment were also taken:

- sediment A: bottom of the tank;
- sediment B: collected from boxes containing some lobsters

On arrival in Glasgow haemolymph from each animal was plated in MIA-media plates at the following dilutions: 1/1, 1/10, 1/100, 1/1000 and hepatopancreas smears were also plated in MIA-media plates. Sediments were re-suspended with 1 ml of *Nephrops* saline solution and supernatants were plated at the dilutions of: 1/1, 1/10, 1/100, 1/1000, 1/10000. Volume plated in the case of haemolymph and sediment was 100 µl. Plates were incubated at 18 °C for 48 h.

Results on the total bacteria counts in haemolymph and sediment supernatants obtained in the plates are shown in table 1.

Table 1. TVC in haemolymph and sediment supernatants obtained in the March sampling. Each haemolymph and sediment supernatant was plated in triplicates. Individual values and not averages are shown in the table.

	Lobster 1	Lobster 2	Lobster 3	Lobster 4	Sediment A	Sediment B
Dilution 1/1	0/1/1	3/2/4	3/1/2	8/17/15		
Dilution 1/10	0/0/1	1/0/0	0/0/0	4/2/1		
Dilution 1/10000					37/57/45	63/79/85

Haemolymph samples contained very few numbers of bacteria while sediment supernatants had very high numbers of bacteria. Hepatopancreas smears showed higher number of bacteria compared to haemolymph although colonies were not counted as they came from a smear (Figure 1).

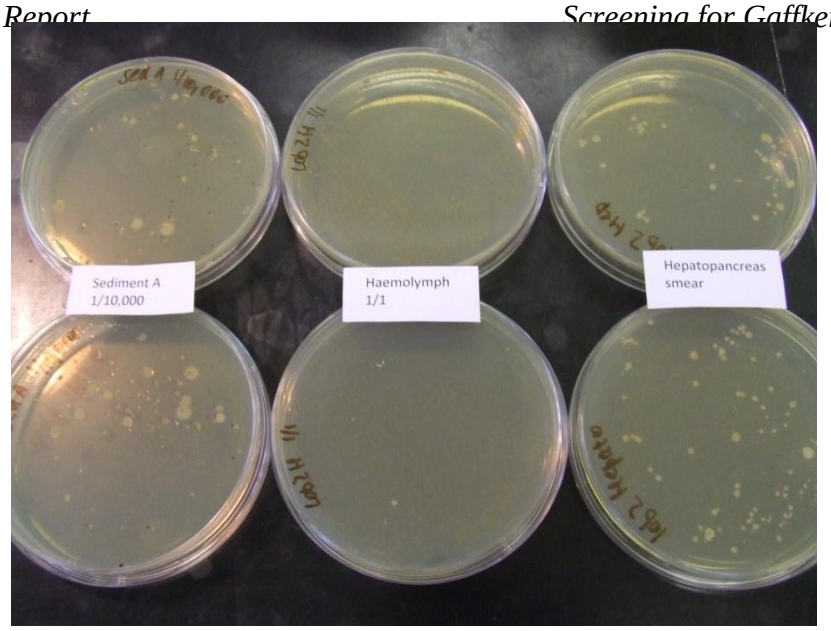


Figure 1. Picture showing MIA plates with colonies obtained from sediment supernatants (dilution 1/10000), haemolymph (dilution 1/1) and from a hepatopancreas smear

From all the colonies obtained in the plates a total of 13 different colonies of haemolymph, 6 colonies from the hepatopancreas and 7 colonies from the sediments supernatants were further studied by doing gram staining and results are summarized in Table 2.

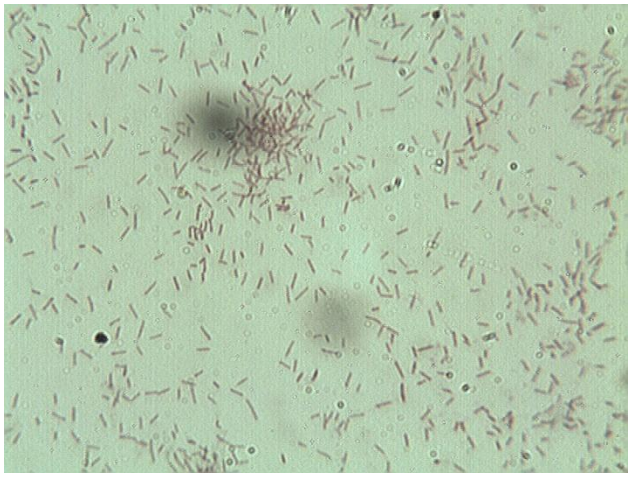
Table 2. Information and gram staining results from selected colonies (March sampling)

Num colony	Shape colony	Colour colony	Size colony	Origin	More info	Gram staining/shape bacteria
1	Round	Orange	M/B	Haemolymph	Lobs 2	Gram negative, rod. Similar to colony 36
2	Round	Cream/trans	M	Haemolymph	Lobs 2	Gram negative, short rod
3	Round	Cream/white	M/B	Haemolymph	Lobs 4	Gram negative, long filamentous rod
4	Round	Yellow	M/B	Haemolymph	Lobs 4	Gram negative, rod
5	Round/Irreg	Orange/yell	B	Haemolymph	Lobs 4	Gram negative, rod
6	Round/Irreg	Trans lucid	M	Haemolymph	Lobs 4	Gram negative, rod
7	Irregular	White	B	Haemolymph	Lobs 4	Gram negative
8	Round	Trans /yellow	B	Haemolymph	Lobs 4	Gram negative
9	Round	Trans lucid	S	Haemolymph	Lobs 4	Gram positive, rod
10	Round	White/Trans	S	Haemolymph	Lobs 1	Gram negative, rod
11	Round	Cream/Trans	M	Haemolymph	Lobs 2	Gram negative, short rod

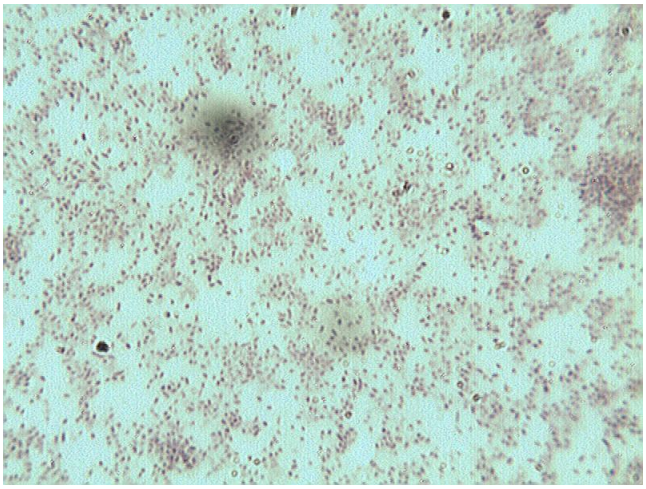
Num colony	Shape colony	Colour colony	Size colony	Origin	More info	Gram staining/shape bacteria
12	Round	Orange	S/M	Haemolymph	Lobs 4	Gram negative, short rod
13	Round/Irreg	Yellow	M	Haemolymph	Lobs 4	Gram negative. Short rod
21	Round	Cream/black	M	Hepatopanc	Lobs 2	Gram negative, short rod
22	Round	Cream	M	Hepatopanc	Lobs 3	Gram negative, ovoid. Similar to colonies 24, 26.
23	Round	Trans lucid	M	Hepatopanc	Lobs 4	Gram negative, rod
24	Round	Cream	S	Hepatopanc	Lobs 1	Gram negative, ovoid. Similar to colonies 22, 26.
25	Round	Cream/Orange	M	Hepatopanc	Lobs 2	Gran negative, rod. Similar to colony 23.
26	Round	Cream	M	Hepatopanc	Lobs 4	Gram negative, ovoid. Similar to colonies 22, 24.
31	Round	Trans/black	M	Sediment	A	Gram negative, rod
32	Round	Cream	M/B	Sediment	A	Gram negative, cocci
33	Round	Yellow/Trans	M/B	Sediment	A	Gram negative, rod
34	Round/Irreg	Orange	B	Sediment	B	Gram negative, filamentous
35	Round	Pink middle	M	Sediment	B	Gram positive, rod. Possibly H_2S producer
36	Round	Orange	M	Sediment	B	Gram negative, rod
37	Round	Yellow	M/S	Sediment	A	Gram negative, rod curved like kidney shape

As none of the selected studies colonies were not gram positive cocci no biochemical studies were performed in any of the colonies from the March sampling. Some of the most common morphologies are shown in Figure 2.

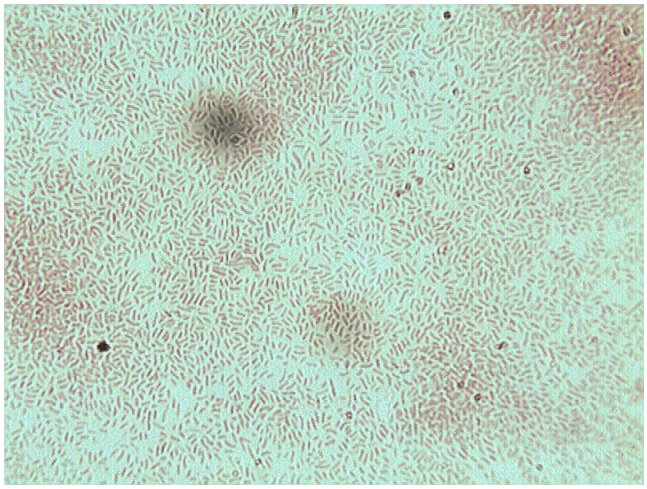
Colony 4



Colony 21



Colony 25



Colony 34

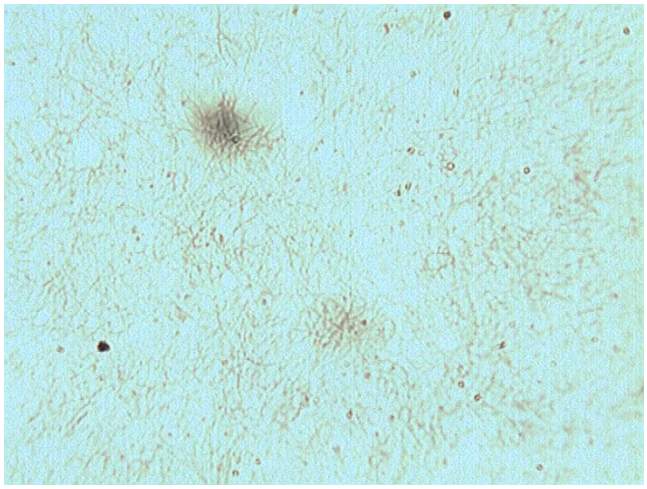


Figure 2. Gram staining slides of colonies 4, 21, 25 and 34 all obtained in the March sampling in a magnification of x1000.

APRIL SAMPLING – On the 22/04/2010 we received 2 lobsters still alive from Tarbert fish farm. Lobster 1 was a female with well developed green gonads. The origin of lobster 1 was West of Scotland. Lobster 2 on the other hand was originally from the East of Scotland, this lobster was also a female with well developed green gonads. None of the lobsters presented signs of disease. Sampling in April was similar to the sampling performed in March. Haemolymph in dilutions 1/1 and 1/10 were plated in TSA-media

plates and we also performed smears of hepatopancreas that were also plated in TSA-media plates. All plates were incubated at 28 °C for 48 h. Furthermore, in this sampling we also performed gram staining of the haemolymph directly in order to establish if by any chance the lobsters had an advance stage of infection that could be detected in this way. Results as expected indicated that these lobsters were free of advance infection as haemolymph gram staining did not show any aggregation of bacteria. Results from the plates indicated that the levels of bacteria were low in haemolymph (Table 3) and also in hepatopancreas.

Table 3. TVC in haemolymph plated samples obtained in the April sampling. Each haemolymph was plated in triplicates. Individual values and not averages are shown in the table.

Haemolymph dilution	Lobster 1-West of Scotland	Lobster 2- East of Scotland
Dilution 1/1	2/1/1	0/0/0
Dilution 1/10	0/0/0	0/0/0

All the colonies obtained from lobster 1 haemolymph were further studied by performing gram staining and results are shown in Table 4. Furthermore, as some more colonies were obtained in the plates from hepatopancreas smears a total of 4 colonies of lobster 1 and 5 colonies of lobster 2 were also further studied.

Table 4. Information and gram staining results from selected colonies (March sampling). In black we highlight colonies were re-cultured and maintained at -80 °C to perform biochemical tests.

Num colony	Shape colony	Colour colony	Size colony	Origin	More info	Gram staining/shape bacteria
40	**Round**	**Yellow**	**M**	**Hepatopancr**	**Lobs 1**	**Gram positive, cocci. Some tetrads, re-culture**
41	Round	Cream/white	M/B	Hepatopancr	Lobs 1	Gram negative, rod
42	Round	Cream/white	B	Hepatopancr	Lobs 1	Gram negative, rod. Similar to colonies 46 and 47.
43	**Round**	**White**	**S**	**Hepatopancr**	**Lobs 1**	**Gram negative, cocci. Re-culture**
44	Round	Orange	M	Hepatopancr	Lobs 2	Gram negative, rod
45	Round	White	M	Hepatopancr	Lobs 2	Gram negative, short rod

46	Round	Cream/white	B	Hepatopancr	Lobs 2	Gram negative, rod. Similar to colonies 42 and 47.
47	Irregular	Cream/white	B	Hepatopancr	Lobs 2	Gram negative, rod. Similar to colonies 42 and 46.
48	Irregular	Trans lucid	M	Hepatopancr	Lobs 2	Gram positive, short rod
49	**Round**	**Yellow**	**M**	**Hepatopancr**	**Lobs 2**	**Gram positive, cocci. Re-culture**
54	Round	Yellow/trans	S	Hepatopancr	Lobs 1	Gram negative, short rod
50	**Round**	**Yellow**	**M**	**Haemolymph**	**Lobs 1**	**Gram positive, cocci. Some tetrads. Re-culture**
51	**Round**	**Yellow**	**M**	**Haemolymph**	**Lobs 1**	**Gram positive, cocci. Some tetrads. Re-culture**
52	**Round**	**White**	**S**	**Haemolymph**	**Lobs 1**	**Gram positive, cocci. Re-culture**
53	Round	Cream/Trans	M	Haemolymph	Lobs 1	Gram negative, short rod

In the April sampling interestingly we found 5 colonies that were gram positive cocci and one more colony that was gram negative cocci. As gram staining sometimes can be confusing we re-cultured all 6 colonies named: 40, 43, 49, 50, 51 and 52 in TSA-media plates and they were stored at -80 °C until the biochemical tests were performed.

Colony 40 **Colony 52**

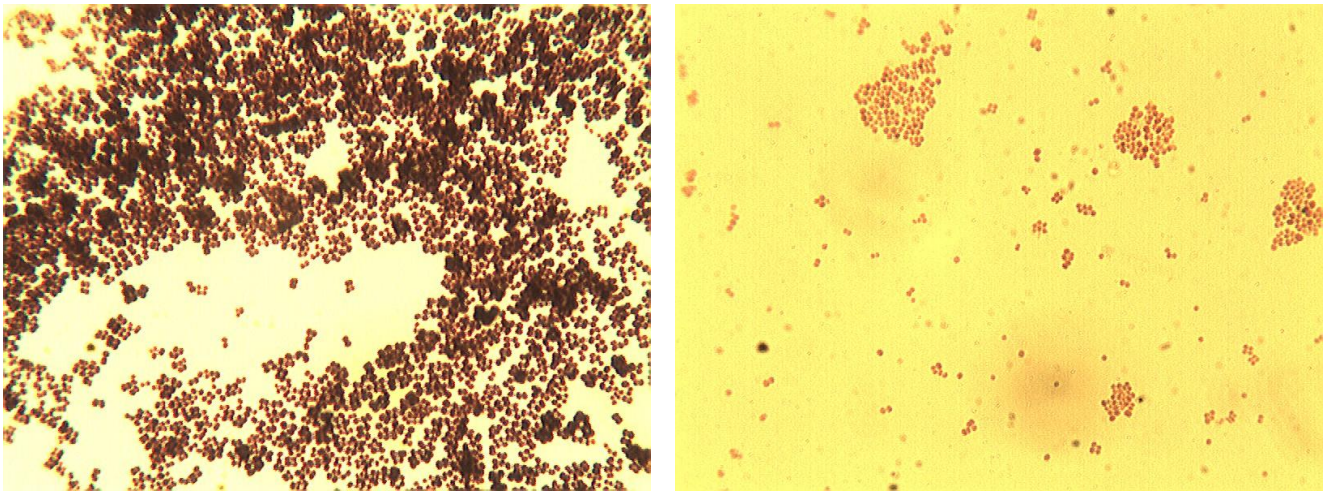


Figure 3. Gram staining slides of colonies 40 and 52 in a magnification of x1000.

Biochemical results from colonies selected in March:

Colonies 40, 43, 50, 52 and the reference strain of *A. viridans* were re-grown in phenylethyl alcohol agar or PEA-media plates as this media is selective for *Staphylococcus* genus (gram positive cocci, includes at least 40 species) while inhibiting gram-negative bacilli. All the selected colonies grew well in this media confirming that the selected colonies were indeed gram positive cocci.

Two biochemical tests were performed in order to further elucidate if any of the gram positive cocci colonies obtained in the April sampling were *A. viridans*:

1) *StrepQuick test*: This a quick test kit that is intended to aid in the identification of gram-positive, catalase-negative cocci as it detects activity of three enzymes: pyroglutamate aminopeptidase (PYR), leucine aminopeptidase (LAP) and esculinase (ESC). Typically, *A. viridans* shown positive results for PYR and negative reactions to LAP and ESC.
2) *Api 20 Strep*: This test is a standardized method that combines 20 biochemical tests. The enzymatic tests (they are contained in microtubes) are inoculated with a dense suspension of bacteria, made from a pure culture. The metabolic end products produced during the inoculation period are used to identify the specie of the bacteria inoculated. Results of the different reactions are monitored after 4 and also 24 h after inoculation.

As shown in Figure 4 when the StrepQuick test was applied to the reference strain of *A. viridans* the results were as reported in the instructions of the kit:

PYR	LAP	ESC
+	-	-

Results from the selected colonies on the StrepQuick test were as follows:

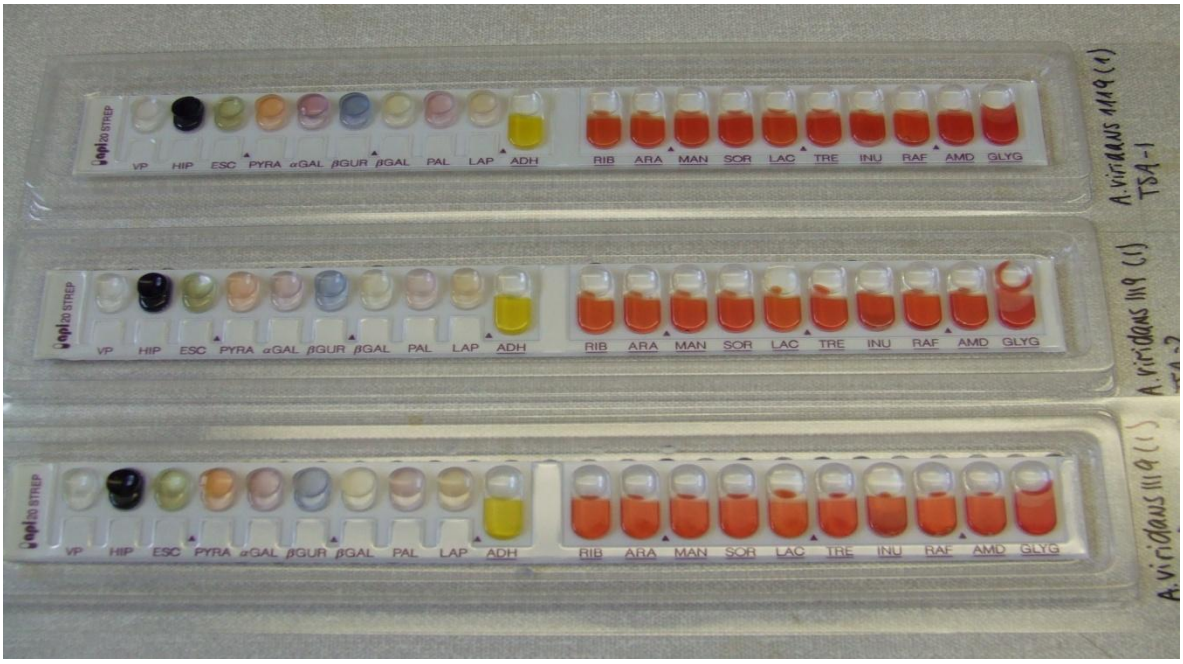
	PYR	LAP	ESC
Colony 40	+	+	-
Colony 43	+	+	-
Colony 50	+	+	-
Colony 52	+	-	-

Therefore, according to the StrepQuick test colony 52 would be the only potential colony that could belong to the species *A. viridans* (Figure 4).

Figure 4. Results from the StrepQuick test kit on colonies 40, 52 and *A. viridans* reference strain.

In this *A. viridans* reference strain we also performed the test Api20 Strep a total of 4 times and using 2 different media to grow the bacteria (TSA and PEA-media plates) in order to see how reliable this test was and to look at the variability between media. In general, the test was reproducible if the same media was used to grow the bacteria, although sometimes it was difficult to judge if the result was positive or negative (Figure 5 and 6).

A)



B)

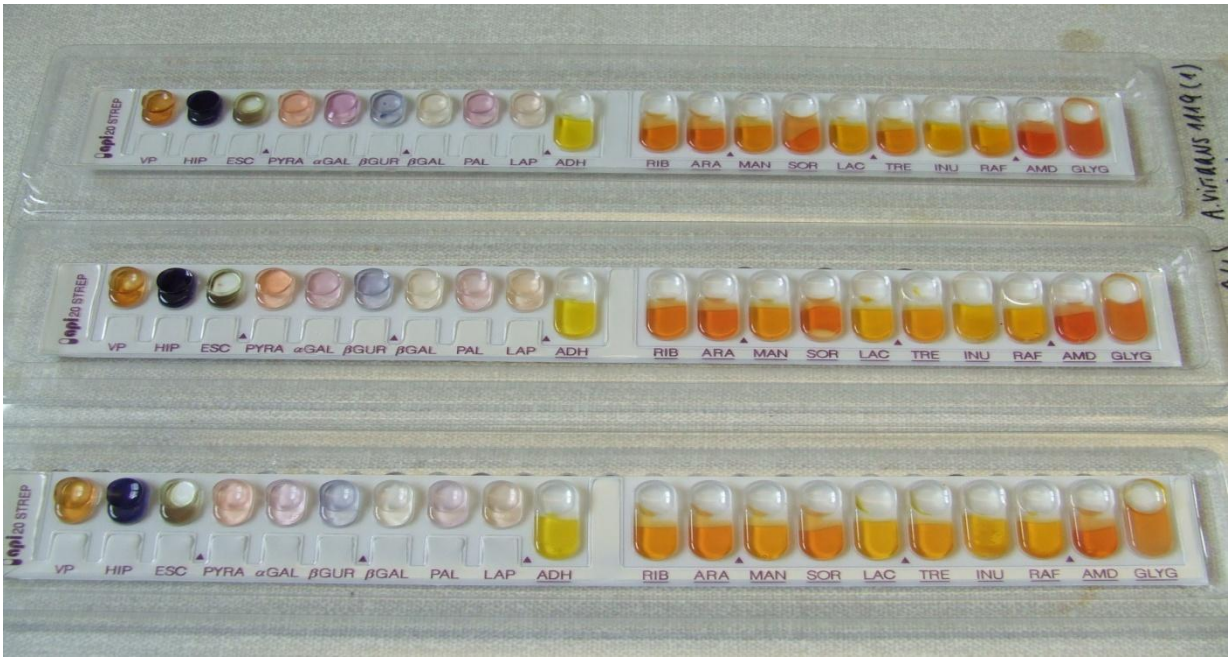
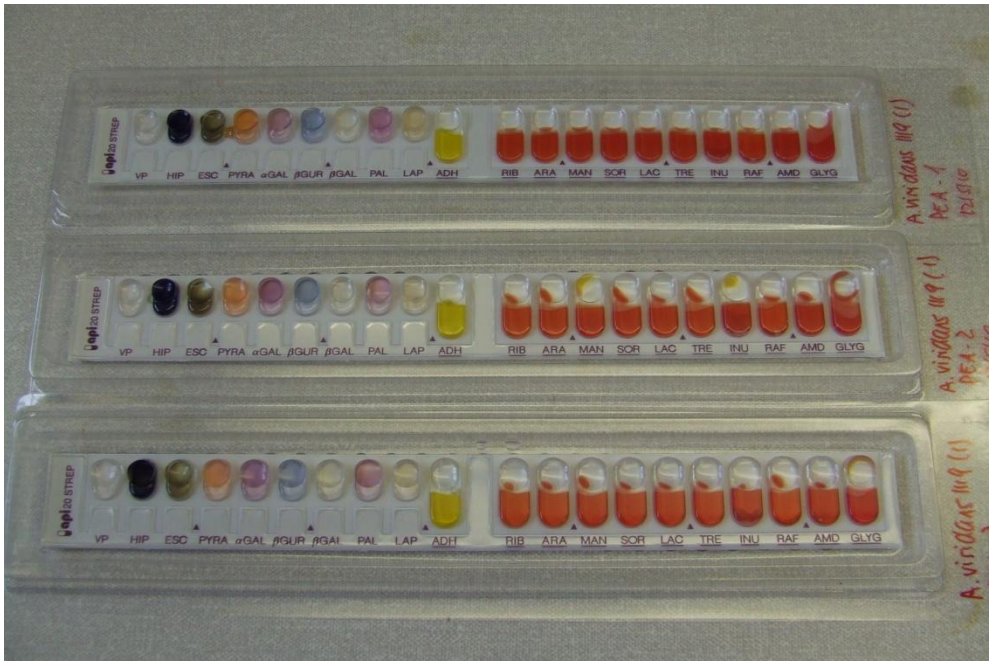


Figure 5. Results from the Api 20 Strep test on *A. viridans* reference strain cultured in TSA plated. A) Results after 4 h and B) Results after 24 h.

A)



B)

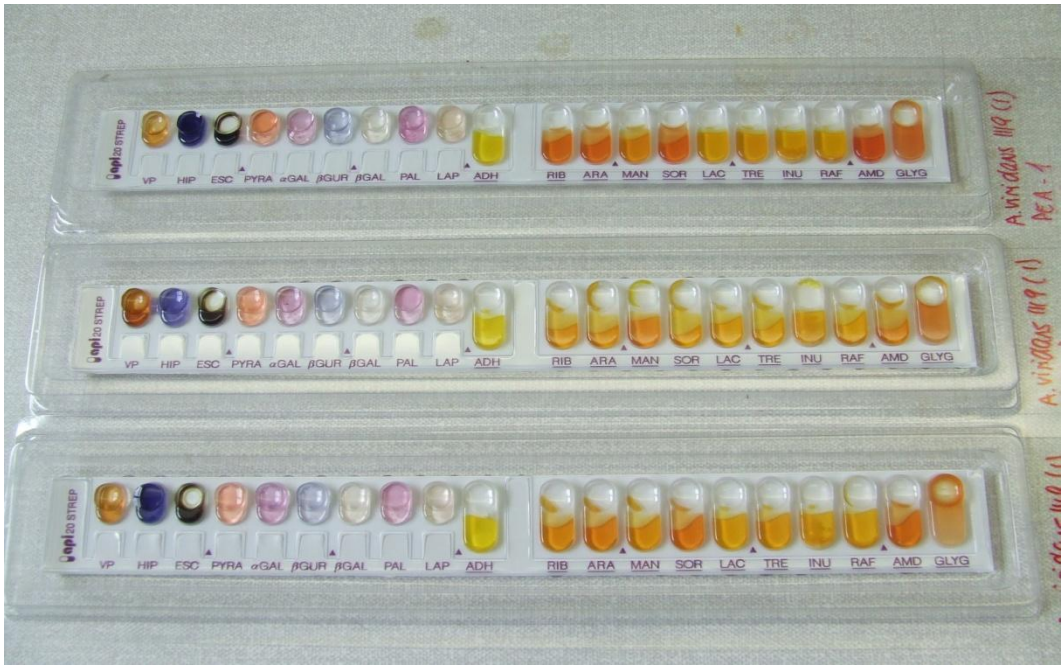


Figure 6. Results from the Api 20 Strep test on *A. viridans* reference strain cultured in PEA plated. A) Results after 4 h and B) Results after 24 h.

Results from Api20 Strep for the reference strain *A. viridians* can be summarized as follows:

Colony: *A. viridans* reference strain 1119
Media: TSA

t1	t2	t3	t4	t5	t6	t7	t8	t9	t10	t11	t12	t13	t14	t15	t16	t17	t18	t19	t20
-	+	-	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-
		-							-	-	-	+	-	+	+	+	+	-	-

Each column represents the results obtained for each of the 20 tests that are performed in the Api20 Strep. In the first line are represented the results obtained after 4 h of inoculation and the second line represents the results obtained after 24 h of inoculation. In yellow are selected the test conditions that were most consistent and reliable.
Profile according to ApiWeb™ was 2406770. The software identified this profile as *A. viridans* 1 with an ID % OF 99.5% and a T of 0.47 indicating that the identification was very good.
This test was also performed in *A. viridans* previously grown in PEA plates. Results on the Api20 Strep in this case were:

Colony: *A. viridans* reference strain 1119
Media: PEA

-	+	+	-	-	+	-	-	-	-	+	+	+	+	+	+	-	+	*	-
		+							-	-	-	+	-	+	+	+	+	-	-

Profile according to ApiWeb™ was 6406751. The software identified this profile as *A. viridans* 1 with an ID % of 99.5% and a T of 0.79 indicating that the accuracy of the identification was very good.

Results from the selected colonies in the month of April were as follows.

Colony: Colony 40
Media: TSA

t1	t2	t3	t4	t5	t6	t7	t8	t9	t10	t11	t12	t13	t14	t15	t16	t17	t18	t19	t20
-	+	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-
		-							-	-	-	-	-	-	-	-	-	-	-

Profile according to ApiWeb™ was 2146771. The software identified this profile as *A. viridans* 1 with an ID % of 96.8% and a T of 0.45 indicating that the accuracy of the identification was good.

Colony: Colony 43
Media: TSA

t1	t2	t3	t4	t5	t6	t7	t8	t9	t10	t11	t12	t13	t14	t15	t16	t17	t18	t19	t20
-	+	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-
		-							-	-	-	-	-	-	-	-	-	-	-

Profile according to ApiWeb™ was 2146771. The software identified this profile as *A. viridans* 1 with an ID % of 96.8% and a T of 0.45 indicating that the accuracy of the identification was good.

Colony: Colony 50
Media: TSA

t1	t2	t3	t4	t5	t6	t7	t8	t9	t10	t11	t12	t13	t14	t15	t16	t17	t18	t19	t20
-	+	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-
		-							-	-	-	-	-	-	-	-	-	-	-

Profile according to ApiWeb™ was 2146771. The software identified this profile as *A. viridans* 1 with an ID % of 96.8% and a T of 0.45 indicating that the accuracy of the identification was good.

Colony: Colony 52
Media: TSA

t1	t2	t3	t4	t5	t6	t7	t8	t9	t10	t11	t12	t13	t14	t15	t16	t17	t18	t19	t20
+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		+							-	-	-	-	-	-	-	-	-	-	-

Profile according to ApiWeb™ was 7000000. The software identified this profile as Listeria sp. with an ID % of 86.1% and a T of 0.64 indicating that the accuracy of the identification was acceptable.

As shown by these results colonies 40, 43 and 50 gave the same results in the biochemical tests performed. Results indicated that all these colonies were possibly *A. viridans* 1 while colony 52 was different and results suggested that this colony could be

from the genus Listeria. However, none of the identifications gave an accuracy of very good or excellent.

MAY SAMPLING – On the 13th of May 2010 we received another 2 lobsters from Tarbert fish farm. Lobster 1 was originally from the East Coast, male with a CL of 92.6 mm while lobster 2 was originally from the West Coast, male with a CL of 97.2 mm. In this sampling haemolymph (un-diluted) and hepatopancreas smears were platted in TSA and also in PEA-media plates. All plates were incubated at 28 °C for 48 h. Total viable counts of bacteria are shown in Table 5 and 6.

Table 5. TVC in haemolymph plated samples obtained in the May sampling. Each haemolymph was plated in tetraplicates. Individual values and not averages are shown in the table.

Haemolymph	Lobster 1-East of Scotland	Lobster 2- West of Scotland
Dilution 1/1 TSA-media	0/0/0/1	0/0/0/1
Dilution 1/1 PEA-media	0/0/0/0	3/0/0/0

Table 6. TVC in hepatopancreas smears plated samples obtained in the May sampling. Each plate contained the bacteria from an individual hepatopancreas smear. Individual values and not averages are shown in the table.

Hepatopancreas smears	Lobster 1-East of Scotland	Lobster 2- West of Scotland
TSA-media	1/2/1/6	12/3/7
PEA-media	0/0/0	2/0/0

Similarly, to previous samplings bacteria numbers were low especially in the haemolymph suggesting that none of the lobsters had an advanced level of infection.

Table 4. Information and gram staining results from selected colonies (May sampling). In black we highlight colonies were re-cultured and maintained at -80 °C to perform biochemical tests.

Num colony	Shape colony	Colour colony	Size colony	Origin	More info	Gram staining/shape bacteria
60	**Irregular**	**White**	**S**	**Hepatopancr**	**Lobs 2**	**Gram staining not clear, small cocci**
61	**Irregular**	**Cream**	**S**	**Hepatopancr**	**Lobs 2**	**Gram staining not clear, small cocci**

62	Round	Cream	B	Hepatopancr	Lobs 1	Gram negative, short rod
63	**Round**	**Yellow**	**S/M**	**Hepatopancr**	**Lobs 1**	**Gram positive, cocci**
64	Round	Cream	M	Hepatopancr	Lobs 2	Gram negative, rod
65	Round	Trans lucid	S	Hepatopancr	Lobs 2	Gram negative, rod
66	Round	Trans /cream	M	Hepatopancr	Lobs 2	Gram negative, short rod
70	**Round**	**Yellow**	**M**	**Haemolymph**	**Lobs 2**	**Gram cocci, cocci, tetrads not clear**
71	Round	Cream	S	Haemolymph	Lobs 1	Gram negative, filamentous rods
72	Round	Trans lucid	S	Haemolymph	Lobs 2	Gram negative, short rod
73	**Round**	**Yellow**	**M/S**	**Haemolymph**	**Lobs 2**	**Gram staining not clear, cocci**
74	**Round**	**Yellow**	**M/S**	**Haemolymph**	**Lobs 2**	**Gram staining not clear, cocci**

From these results it is important to mention that colonies 70, 73 and 74 were very similar.

Biochemical results from colonies selected in May:

All the selected colonies were re-grown in PEA-media plates to confirm that they were indeed gram positive cocci. Interestingly, colonies 60 and 61 did not grow in PEA plates and no more studies were performed on them while colonies 63, 70, 73 and 74 grew well in PEA-media plates confirming that they were indeed gram positive cocci.

When we performed the StrepQuick in the selected colonies the following results were obtained, see also Figure 7:

	PYR	LAP	ESC
Colony 63	+	+	-
Colony 70	+	+	-
Colony 73	+	+	-
Colony 74	-	+	-

Therefore, according to the StrepQuick test kit none of the selected colonies in May could be *A. viridans* as they all gave a positive result for LAP.

Figure 7. Results from the StrepQuick test kit on colonies 63, 70, 73 and 74. Please note that in colony 74 the results for PYR as negative as the colour in the circle is not d cherry red but a salmon colour.

Nevertheless, we also performed the test Api20 Strep in these selected colonies are compared the results with the ones obtained with *A. viridans* reference strain.

Colony: Colony 63
Media: TSA

-	+	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-
		-							-	*	*	*	*	-	+	-	-	+	-

Profile according to ApiWeb™ was 2146771. The software identified this profile as *A. viridans* 1 with an ID % of 96.8% and a T of 0.45 indicating that the accuracy of the identification was good.

Colony: Colony 63
Media: PEA

-	+	-	+	-	-	-	+	+	-	+	+	+	+	+	+	-	+	+	-
		-							-	*	-	-	-	-	+	-	-	-	-

Profile according to ApiWeb™ was 2166751. The software identified that the closest possibility to this profile was *A. viridans* 1 but with an unacceptable accuracy.

Colony: Colony 70
Media: PEA

-	+	-	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	-
		-							-	-	-	-	-	-	+	-	-	*	-

Profile according to ApiWeb™ was 2146771. The software identified this profile as *A. viridans* 1 with an ID % of 96.8% and a T of 0.45 indicating that the accuracy of the identification was good.

Colony: Colony 73
Media: PEA

-	+	-	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	-
		-							-	-	-	-	-	-	+	-	-	-	-

Profile according to ApiWeb™ was 2146771. The software identified this profile as *A. viridans* 1 with an ID % of 96.8% and a T of 0.45 indicating that the accuracy of the identification was good.

Colony: Colony 74
Media: PEA

-	+	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	-
		-							-	-	+	-	+	+	+	-	+	+	-

Profile according to ApiWeb™ was 2066771. The software identified that the closest possibility to this profile was *A. viridans* 1 but with an unacceptable accuracy.

Similarly to the situation in the April sampling it was not possible to conclude that any of the selected colonies were *A. viridians* although colonies 63, 70 and 73 had strong possibilities to be *A. viridans*.

JUNE SAMPLING – On the 22nd of June 2010 we received 2 lobsters from Tarbert fish farm. Lobster 1 was female with a CL of 89.0 mm with green dark developed gonads (red band/ 1 claw missing) while lobster 2 was fe male with a CL of 76.2 mm with pale green gonads not so developed (white band/ 1 claw missing). In this sampling haemolymph (un-diluted) and hepatopancreas smears were plated in TSA and also in PEA-media plates. All plates were incubated at 28 °C for 48 h. Total viable counts of bacteria are shown in Table 7 and 8. We also performed a gram staining of haemolymph smears. Total viable counts of bacteria are shown in Table 7 and 8 and similarly to previous sampling, bacteria numbers were low. On the other hand, gram staining of haemolymph smears did not show any indication of an advanced stage of infection.

Table 7. TVC in haemolymph plated samples obtained in the June sampling. Each haemolymph was plated in quadruplicate. Individual values and not averages are shown in the table.

Haemolymph	Lobster 1	Lobster 2
Dilution 1/1 TSA-media	0/0/0/0	1/1/0/0
Dilution 1/1 PEA-media	0/0/0/0	0/0/0/0

Table 8. TVC in hepatopancreas smears plated samples obtained in the June sampling. Each plate contained the bacteria from an individual hepatopancreas smear. Individual values and not averages are shown in the table.

Hepatopancreas smears	Lobster 1	Lobster 2
TSA-media	0/0/0	1/1/2
PEA-media	0/0/0	0/0/0

Gram staining and morphology of the colonies obtained the June sampling are shown in Table 9.

Table 9. Information and gram staining results from selected colonies (June sampling). In black we highlight colonies were re-cultured and maintained at -80 °C to perform biochemical tests.

Num colony	Shape colony	Colour colony	Size colony	Origin	More info	Gram staining/shape bacteria
81	**Round**	**White**	**M/S**	**Haemolymph**	**Lobs 1**	**From TSA plate/Gram negative, cocci, no tetrad**
82	**Round**	**Orange**	**B**	**Haemolymph**	**Lobs 2**	**From TSA plate/Gram positive, cocci, no tetrads**
83	**Round**	**Cream**	**B**	**Hepatopancr**	**Lobs 2**	**From TSA plate/Gram positive, cocci**
84	Round	Cream	M	Hepatopancr	Lobs 2	Gram negative, short rods
85	Round	Cream	M	Hepatopancr	Lobs 2	Gram negative, rod
86	Round	Trans lucid	S	Hepatopancr	Lobs 2	Gram negative, short rod

From this sampling the colonies 81, 82 and 83 were selected for further biochemical analysis. Results from the StrepQuick in the selected colonies are shown here and also in Figure 8:

	PYR	LAP	ESC
Colony 81	+	-	-
Colony 82	-	-	-
Colony 83	-	+	-

Figure 8. Results from the StrepQuick test kit on colonies 81, 82, and 83.

Therefore according to this test the only colony that could be *A. viridians* would be colony 81. Results from the Api20 Strep are shown here:

Colony: Colony 81
Media: TSA

+	+	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-							+	-	-	-	-	-	-	-	-	-	-

Profile according to ApiWeb™ was 3100000. The software identified this profile as *A. viridans* 2 with an ID % of 94.4% and a T of 0.67 indicating that the accuracy of the identification was good.

Colony: Colony 82
Media: TSA

+	+	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	+
		+/-							+	+	-	+	-	-	+	-	+	-	-

Profile according to ApiWeb™ was 3446753. The software identified that the closest possibility to this profile was *A. viridans* 1 but with an unacceptable accuracy.

Colony: Colony 83
Media: TSA

-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	-	+	+	-
		-							-	-	-	-	-	-	-	-	-	-	-

Profile according to ApiWeb™ was 0046751. The software identified this profile as *A. viridans* 2 with an ID % of 93.0% and a T of 0.49 indicating that the accuracy of the identification was good.

AUGUST SAMPLING – On the 8th of August 2010 we received 3 lobsters we proceed with some in situ sampling in Tarbert fish farm. We collected 3 lobsters and also some water samples from one of the 2 tanks were lobsters were kept. Lobster 1 was male with a CL of 89.6 mm (1 red band; stocked 2-3 weeks ago and placed in a tray); lobster 2 was male with a CL of 95.5 mm (2 red bands; stocked 1 week ago; right tank and not placed in a tray) and finally lobster 3 was a dead male which was a new comer that had not survived the transport. Furthermore, 4 water samples were collected from one of the tanks (see Figure 9).

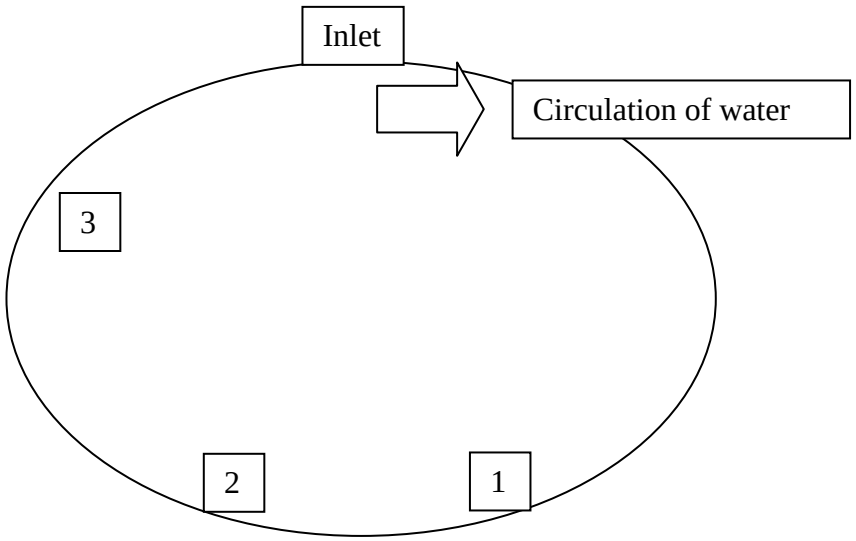


Figure 9. Location of the water samples collected in one of the tanks. Note that inlet water was clear while samples 1, 2 and 3 had scum.

In this sampling haemolymph (un-diluted), water un-diluted or diluted and hepatopancreas smears were plated in TSA and also in PEA-media plates. All plates were incubated at 28 °C for 48 h. Total viable counts of bacteria are shown in Table 10, 11 and 12. We also performed a gram staining of haemolymph smears. Total viable counts of bacteria in haemolypmh and hepatopancreas from lobster 1 and 2 were low while higher number were obtained in lobster 3 possibly due to the fact that the animal was dead. On the other hand, gram staining of haemolymph smears did not show any indication of an advanced stage of infection.

Table 10. TVC in haemolymph plated samples obtained in the August sampling. Each haemolymph was plated in tetraplicates. Individual values and not averages are shown in the table.

Haemolymph	Lobster 1	Lobster 2	Lobster 3
Dilution 1/1 TSA-media	1/1/7	0/0/0	Dead, not available
Dilution 1/1 PEA-media	0/0/1	0/0/1	Dead, not available

Table 11. TVC in hepatopancreas smears plated samples obtained in the August sampling. Each plate contained the bacteria from an individual hepatopancreas smear. Individual values and not averages are shown in the table.

Hepatopancreas smears	Lobster 1	Lobster 2	Lobster 3
TSA-media	9/0/4	5/6/4	-
PEA-media	0/0/0	1/2/13	18/54/14

Table 12. TVC in water samples obtained in the August sampling. Each plate contained the bacteria from an individual hepatopancreas smear. Individual values and not averages are shown in the table.

Hepatopancreas smears	Inlet 1	Water 1	Water 2	Water 3
TSA-media (1/1)	37/95			
PEA-media (1/1)	4/4			
TSA-media (1/10)				
PEA-media (1/10)		13/5/1	13/15	13/10
TSA-media (1/100)				
PEA-media (1/100)		1/2	2/3	2/0
TSA-media (1/1000)		53/43	10/15	35/21
PEA-media (1/1000)				

Characteristics of the bacteria obtained are shown in Table 13.

Table 9. Information and gram staining results from selected colonies (August sampling). In black we highlight colonies were re-cultured and maintained at -80 °C to perform biochemical tests.

Num colony	Shape colony	Colour colony	Size colony	Origin	More info	Gram staining/shape bacteria
90	**Round**	**Yellow**	**M**	**Haemolymph**	**Lobs 1**	**From PEA plate/Gram positive, cocci**
91	**Round**	**White**	**M**	**Haemolymph**	**Lobs 2**	**From PEA plate/Gram positive, cocci, no tetrads**
92	**Round**	**White**	**M**	**Haemolymph**	**Lobs 1**	**From TSA plate/Gram positive, cocci, no tetrads**
93	Round	Yellow	M/S	Haemolymph	Lobs 1	From TSA plate/Gram negative, short rods
94	Round	Cream/Trans	M	Hepatopancr	Lobs 3	From PEA plate/Filamentous bacteria
95	Round	White	M	Hepatopancr	Lobs 3	From PEA plate/Gram positive, cocci
96	Round	Trans/white centre	M	Hepatopancr	Lobs 3	From PEA plate/Staining not conclusive, rods
98	Round	Cream	M	Hepatopancr	Lobs 2	From PEA plate/Gram negative, rods
99	**Round**	**White**	**M**	**Inlet H_2O**	**Lobs 2**	**From PEA plate/Gram positive, cocci, no tetrads**
100	**Round**	**Cream dark**	**M**	**Inlet H_2O**	**Lobs 2**	**From PEA plate/Gram positive, cocci, no tetrads**
101	Round	Cream	M	Inlet H_2O	Lobs 2	From PEA plate/Staining not conclusive, rods
102	Round	Trans lucid	M/S	1-H_2O	Lobs 2	From TSA plate/Gram negative, rods
103	Irregular	White	B	3- H_2O	Lobs 2	From PEA plate/Gram positive, cocci, no tetrads

Colonies selected for biochemical analysis were 90, 91, 92, 99 and 100. Results from the StrepQuick in the selected colonies are shown here and also in Figure 10:

	PYR	LAP	ESC
Colony 90	+	+	-
Colony 91	+	-	-
Colony 92	+	-	-
Colony 99	-	-	-
Colony 100	+	-	-

Figure 10. Results from the StrepQuick test kit on some of the colonies selected from the August sampling (90, 100 and 99 colonies).

Therefore according to this test colonies that could be *A. viridians* would be colony 91,92 and 100. Results from the Api20 Strep are shown here:

Colony: Colony 90
Media: PEA

-	+	-	+	-	-	-	-	+	-	+	+	-	+	+	+	-	+	-	+
		-							-	-	-	-	-	+	+	-	-	-	+

Profile according to ApiWeb™ was 2146652. The software identified that the closest possibility to this profile was *A. viridans* 1 but with an unacceptable accuracy.

Colony: Colony 91
Media: PEA

+	+	-	+	-	-	-	+	-/+	-	+	+	-	-	-	-	-	-	-	-
		-							+	-	-	-	-	-	-	-	-	-	-

Profile according to ApiWeb™ was 3126000. The software identified that the closest possibility to this profile was *A. viridans* 2 but with an unacceptable accuracy.

Colony: Colony 92
Media: PEA

+	+	-	+	-	-	-	+	-/+	-	+	+	-	-	-	-	-	-	-	-
		-							+	-	-	-	-	-	-	-	-	-	-

Profile according to ApiWeb™ was 3126000. The software identified that the closest possibility to this profile was *A. viridans* 2 but with an unacceptable accuracy.

Colony: Colony 99
Media: PEA

+	+	-	-	-	-	-	+	-/+	-	+	+	-	+	+	-	+	+	-	-
		-							+	-	-	-	-	-	-	-	-	-	-

Profile according to ApiWeb™ was 306660. The software identified that the closest possibility to this profile was *A. viridans* 1 but with an unacceptable accuracy.

Colony: Colony 100
Media: PEA

-	+	+	+	-	-	-	+	+	-	+	+	-	+	+	+	-	+	+	+
		+							-	-	-	-	-	-	+	-	-	-	-

Profile according to ApiWeb™ was 6166651. The software identified that the closest possibility to this profile was *A. viridans* 1 but with an unacceptable accuracy.

In summary, the colonies suspected to be *A. viridans* collected through this project were as follows:

Table 10. Resume of the gram positive cocci bacterial colonies selected for further work. In black we highlight colonies that were similar to A. viridians in the StrepQuick test.

Num colony	Shape /size colony	Colour colony	Sample type	TSA growth	PEA growth	StrepQuick	Api20 Strep ID.
40	Round/M	Yellow	Hepato	+	+	+/+/-	*A. viridans* 1 good
43	Round/S	White	Hepato	+	+	+/+/-	*A. viridans* 1 good
50	Round/M	Yellow	Haemo	+	+	+/+/-	*A. viridans* 1 good
52	Round/S	White	Haemo	+	+	+/-/-	*Listeria* sp. good
63	Round/M	Yellow	Hepato	+	+	+/+/-	*A. viridans* 1 good
70	Round/M	Yellow	Haemo	+	+	+/+/-	*A. viridans* 1 good
73	Round/M-S	Yellow	Haemo	+	+	+/+/-	*A. viridans* 1 good
74	Round/M-S	Yellow	Haemo	+	+	-/+/-	*A. viridans* 1 unacceptable
81	Round/M-S	White	Haemo	+	+	+/-/-	*A. viridans* 2 good
82	Round/B	Yellow	Haemo	+	+	-/-/-	*A. viridans* 1 unacceptable
83	Round/B	Cream	Hepato	+	-	-/+/-	*A. viridans* 2 good

90	Round/M	Yellow	Haemo	+	+	+/+/-	*A. viridans* 1 unacceptable
91	Round/M	White	Haemo	+	+	**+/-/-**	*A. viridans* 2 unacceptable
92	Round/M	White	Haemo	+	+	**+/-/-**	*A. viridans* 2 unacceptable
99	Round/M	White	H_2O	+	+	**-/-/-**	*A. viridans* 1 unacceptable
100	Round/M	Cream	H_2O	+	+	**+/-/-**	*A. viridans* 1 unacceptable

Therefore, although some colonies had a high potential to be *A. viridans* none of the selected colonies gave an 'excellent' or 'very good' accuracy according to the biochemical tests. For this reason, it was important to confirm the identity of the different colonies in order to identify which of the colonies were indeed *A. viridans* and therefore to identify the most appropriate identification method to be used in the future. For this reason the colonies shown in Table 10 were re-grown in PEA and TSA plates and DNA was extracted to be able to do a molecular characterization and identification of the colonies. However, there was a technical problem when trying to amplify the DNA obtained from the colonies. The methodology used did not only amplify the DNA from the colonies but we also had a contamination issue as shown in Figure 11.

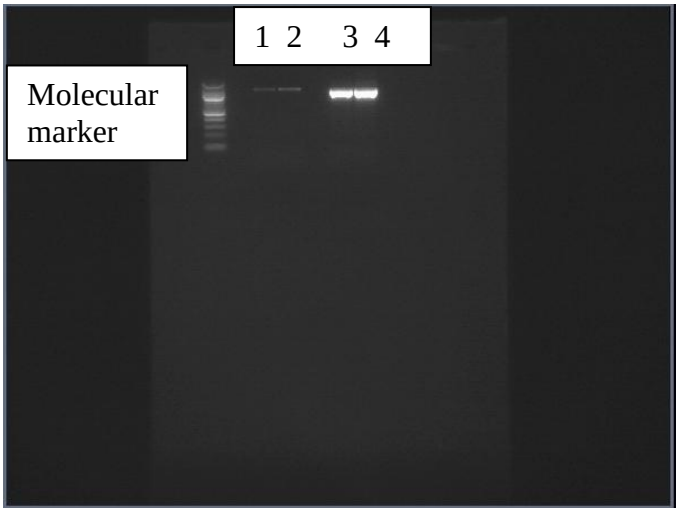


Figure 11.Agarose gel showing an amplified band in the water or blank (lines 1, 2) and also in the *A. viridians* reference strain or positive control (lines 3, 4).

Therefore, although it was possible to obtain and amplify the DNA from all the selected colonies it was not possible to proceed with the sequencing as the negative control indicated that following this protocol led to a contamination of the samples. This was the case following the methodology indicated in Materials and Methods section and also after treating the samples prior to the PCR amplification with 'shrimp nuclease'.

Conclusions and Outlook

- During the period of this project there was no indication of a heavy infection of *A. viridans* in Tarbert fish farm. This was supported by a low mortality (data given by the company) and by the microbiological results of the present work.
- The microbiological data indicated that although there was no outbreak during this investigation it is possible that some of the sampled lobster had low numbers of *A. viridians*.
- From a methodological point of view it would be recommended to do further work in order to sequence and identify molecularly the selected colonies. This step would allow to design and recommend the most suitable tests for future determination of *A. viridans*.
- From the bibliographical data it would appear that the key parameter to control *A. viridians* would be a reduced temperature in the tanks.
- Therefore, it is recommended to cool the water in the tanks.

Acknowledgements

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