EXTRACTION OF LIPOPOLYSACCHARIDES FROM *Vibrio anguillarum*: BUTAN-1-OL METHOD

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Aim of the PhD

• Serotyping *Vibrio anguillarum* using monoclonal antibodies.

• Estimating the susceptibility of marine species to *Vibrio anguillarum*.

• Developing a vaccine using the most pathogenic strains of *Vibrio anguillarum*. 
Structure of Lipopolysaccharides

- Endotoxin on the surface of the outer membrane of Gram-negative bacteria.
LPS extraction

- The original technique from Westphal et al in 1952.
- Relies on the solubility of protein in phenol and LPS/other polysaccharides in water.

An alternative method

- Morrison and Leive (USA), 1975 used Butan-1-ol and presented results which they claim were better than the extraction with Hot-Phenol.

- This method is described a milder method for the isolation of LPS from Gram-negative bacteria.

- Technique has never been widely used
  - LPS Butanol 1,310 hits
  - LPS Phenol 14,700 hits

Vibrio anguillarum growth media and collection

- Vibrio anguillarum from different serotypes (O1, O2α and O2β) grown on 13.5 cm Agar dishes.
- Cellophane sheet layered on top of TSA + 2% NaCl.
- Grown for 4 days at 22 °C.
Application of the procedure

- Equal volume Butan-1-ol, centrifuge 35000g for 20 min.
- Purify (filter) and concentrate.
- Proteinase K (O/N) and nuclease mix, boil.
- Butanol extract, purify (filter), and concentrate.
- Lyophilise.
Silver staining and physical properties

- The yield of LPS collected is similar to the hot-phenol method (10-50 mg).
- Banding pattern of proteinase K treated and butanol extracted LPS similar.
Western blot and monoclonal antibody production

- Western blot using specific monoclonal antibody of butanol extracted LPS along whole bacteria Proteinase K treated.

- The antigenicity is retained with this method.
Macrophage priming

Salmon HK cells 24+24h butanol LPS
Conclusion

• Butan-1-ol is a safe and efficient alternative to phenol for the extraction of lipopolysaccharides of *Vibrio anguillarum*.

• The structure is corresponding with the whole bacteria LPS, the yield and purity are similar to the original method.

• The antigenicity is conserved.

• The priming of macrophages can be achieved with this extraction procedure.
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Phagocytosis of Vibrio anguillarum by Cod (Gadus morhua L.) leukocytes: Live Vs Dead Bacteria.

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Abstract
Most of the studies using flow cytometry for investigating fish immune cell interactions with bacteria use dead bacteria. We present here a preliminary study using live bacteria and show the importance of this to get a more relevant picture especially regarding the respiratory burst produced as a consequence of Vibrio anguillarum phagocytosis by cod leukocytes.

Introduction
Cod is one of the major fish species showing a great potential for development around the Northern hemisphere. Most of the parameters leading to successful aquaculture sector are especially regarding the respiratory burst produced as a consequence of interactions with bacteria use dead bacteria. We present here a preliminary study using live bacteria and show the importance of this to get a more relevant picture especially regarding the respiratory burst produced as a consequence of Vibrio anguillarum phagocytosis by cod leukocytes.

Material and methods
Different isolates of Vibrio anguillarum were grown in TSB+NaCl overnight with some killed overnight in 0.4% formalin. The bacteria were stained with FITC (0.05 mg.mL⁻¹) for 2 h at RT. They were then washed several times and diluted to 5 x 10⁶ c/fmL⁻¹ in L15 with L-glutamine, Pen/Strep and 5% FCS and left overnight. Phagocytosis took place for 2 h at 12 °C in the same media without Pen/Strep with a ratio of bacteria to leukocytes of 20:1, the cells were then washed and analysed on a FACS Calibur.

Results

<table>
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<tr>
<th>Isolates</th>
<th>% of Leukocytes producing a respiratory burst (n=2)</th>
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<tbody>
<tr>
<td>VA53: untyped</td>
<td>35%</td>
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<tr>
<td>VIB2: O2α</td>
<td>70%</td>
</tr>
<tr>
<td>VIB102: O2β</td>
<td>80%</td>
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Conclusion
Phagocytosis and respiratory burst are two of the main innate defence mechanisms that can prevent establishment of a pathogen within the host and produce a disease state. The first lines of defence are extremely important in the clearance of bacteria before they have had time to overcome the immune system. Bacteria have evolved a very broad range of methods to avoid this complex system.

We clearly see here that different serotypes from Vibrio anguillarum (O2α, O2β and untyped) behave differently with respect to inhibition of phagocytosis and respiratory burst. The most pathogenic serotype from this study appeared to be O2β (VIB102) which is in agreement with the isolates reported during natural outbreaks. A difference could also be observed regarding the respiratory burst between live and dead bacteria which could imply that live V. anguillarum are actively inhibiting the production of oxygen radicals in cod. With reference to phagocytosis little difference was observed between live and dead bacteria, which validates the studies using dead bacteria for phagocytic studies with this pathogen.

These preliminary results indicates that using live bacteria draws a clearer picture of possible interactions of Vibrio anguillarum with its host and that further investigation is needed to confirm those results.