PROJECT REPORT: Determining the prevalence of shellfish vibrio pathogens by screening for virulence genes in bacterial isolates

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Bacteria of the genus *Vibrio* are abundant in aquatic environments (Thomson *et al*., 2004). Diversity and virulence of vibrios has been found to increase with water temperature (Beaz-Hidalgo *et al*., 2010; Wendling *et al*., 2014), favouring species which can cause mortality in a range of marine taxa. Pathogenic vibrios are considered to play a significant role in summer mortality of oysters in France (Garnier *et al*., 2007).
In the last 5 years, a number of bacteria have been isolated from shellfish haemolymph, as part of diagnostic investigations into shellfish mortalities by Marine Scotland Science (MSS), the National Reference Laboratory for shellfish health. Because of the work involved to achieve reliable identification, only a limited number of isolates from primary cultures were selected for follow-up tests. In some cases, more abundant isolates in cultures from affected shellfish were sequenced and identified as *Vibrio aestuarianus* and a putative biovar II *V. splendidus*, confirming that these known pathogens can exist in the Scottish marine environment. What is not known is the relative prevalence of these pathogens, nor what proportion of the other vibrios isolated from sampled shellfish may also carry functional virulence markers.
This work was undertaken as an Honours Degree project by Ambre Chapuis, Robert Gordon University, Aberdeen, to develop a tool to screen directly for virulence genes associated with *V. aestuarianus* and *V. splendidus* (Le Roux et al. 2007; Duperthuy et al. 2011) in bacteria isolated from shellfish.

Objectives:

1) Validate PCR assay for each virulence gene using DNA from confirmed *V. splendidus* and *V. aestuarianus* isolates, and from non-vibrio bacteria.
2) Screen 40 vibrio isolates from the MSS culture collection using validated PCR assays and cross-reference against sequence data.

Experimental approach:
Genomic DNA had previously been extracted from bacteria isolated from diagnostic cases - 45 isolates from shellfish and 16 isolates from fish. These bacteria had been identified to species level using routine phenotypical and biochemical methods, as well as sequencing of *gyrB* and *rpoB* genes from fish isolates and *recA*, *rpoB* and *atpA* genes from shellfish isolates (Stagg *et al*. in preparation). Two virulence genes were targeted for *V. splendidus*, the metalloprotease, *vsm*, and the outer membrance protein, *ompU*, using primers designed in this study. For *V. aestuarianus*, the metalloprotease gene, *vam*, was amplified using previously published primers (DeDecker *et al*. 2013). In addition, DNA extracted from *V. tasmaniensis* strain LGP32 (kindly provided by F. Le Roux) was used as positive control in *vsm* and *ompU* amplifications.
Each primer pair was first tested individually against a subset of the shellfish isolates, and amplification conditions were adjusted to improve specificity and yield. The identity of representative amplification products was confirmed by sequencing. The primer pairs with the greatest specificity and yield were chosen for screening all of the shellfish isolates. Separate PCR reactions were carried out for each of the 3 virulence genes. The specificity of the primers for shellfish-associated vibrios was tested by screening against the 16 fish isolates.

Results and conclusions:

***ompU* gene**

The primer pair used for *ompU* strongly amplified products from 20/45 shellfish isolates. Faint products of varying molecular weight were also observed for 9 isolates which were considered as non-specific.

Screening of fish bacterial isolates with ompU primers resulted in amplification of an *ompU* gene from *Aliivibrio wodanis* (1 isolate) and a glutamate synthetase from *Aeromonas salmonicida* (2 isolates).

Further work: Primers for amplification of the *ompU* gene will be redesigned, based on the sequences obtained. This primer pair will be checked for improved specificity against the shellfish and fish isolates. If successful, the specificity will then be tested against non-shellfish *V. splendidus* isolates

***vsm* gene**

Using *vsm* primers, a single, strong product of approximately 350 bp was obtained from 30/45 shellfish isolates. No amplification of fish isolates was observed although strong products were obtained from positive control DNA.

Further work: the specificity of this primer pair will be tested against non-shellfish *V. splendidus* isolates.

***vam* gene**

Using the *vam* primer pair resulted in a strong product of approximately 150 bp from 2 isolates previously identified as *V. aestuarianus.* Faint products of this molecular weight were also obtained from an additional 6/45 shellfish isolates, while faint products of 400-600 bp were obtained from 10/45 isolates. No amplification of fish isolates was observed.

Further work: modification of the thermal cycling conditions will be tested to determine if specificity can be improved.

Some discrepancies have been found between the species identities suggested by the *vsm* and *ompU* virulence genes compared with the housekeeping genes. Increased specificity of the virulence gene primers may improve the agreement between the two sets of information. However, massive lateral gene transfer among *V. splendidus* has been indicated by Nasfi *et al.* (2015) and further work will be required to establish whether specific virulence gene profiles can be correlated with a particular *V. splendidus* strain.

MASTS funding (£844) was used to purchase *Taq* polymerase, molecular weight ladders, primers and DNA clean-up reagents as well as paying for sequencing of amplification products at the Dundee Sequencing and Services Unit.

Reagent/Process	Cost
Taq	204
Primers	88
DNA ladder	116
Extn of ctrl DNA	76
Sequencing	360
Total	844

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