



European Project SUDEVAB

Work package 4 - Pathology

- Health status of cultured abalone, experimental pathology and development of diagnosis tools -

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Objectives of Work Package 4 - Pathology

Task 1: Definition of the **health status** of cultured abalone from FRHAL

Task 2: Experimental pathology - Susceptibility of the European abalone *Haliotis tuberculata* to :

- ✓ different bacterial strains belonging to the genus *Vibrio*
- ✓ the parasite *Perkinsus olseni*
- ✓ Ostreid herpesvirus 1 (OsHV-1)

Task 4: Development of specific tools for the diagnosis of *Vibrio harveyi*

Task 1 - Health status of abalone



Step 1 : Abalone collection



50 animals of two batches for transfers were collected (2 cm in ponds and 4 cm from open sea)

Step 2 : Bacterial analysis

Objective : Research of *Vibrio harveyi* known to be responsible of mortality outbreaks of European abalone in France, since 1997

Step 3 : Histological analysis

Objective: Research of others pathogens or disease signs, in particular *Candidatus xenohaliotis californiensis* already detected in Spain and Irland.

Task 1 - Health status of abalone

Bacterial analysis: searching for *Vibrio harveyi*

Methodology

- Dissection of different organs : gill, foot, gastric gland, and haemolymph (50 animals from each batch)
- Preparation of tissue homogenates from different organs
- Bacterial culture on specific medium for *Vibrio* species (TCBS medium)
- Specific PCR targeting the ToxR gene for colonies with a phenotype similar to *V. harveyi*

Results

- No detection of *Vibrio harveyi* in abalone from France Haliotis farm (100 animals analysed)

→ The remaining animals were fixed in Davidson's fluid for histological analysis

Task 1 - Health status of abalone

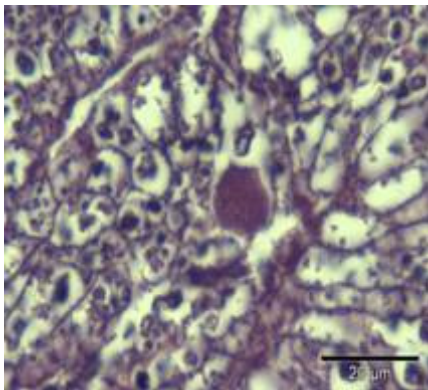
Histological examination

Methodology



- Histological sections were prepared in order to analyse each organ of animals: *gill, nerve, muscle, kidney, intestine, stomach, digestive diverticulum, gastric gland, and gonad*)
- A total of 200 histological slides (3 μm) were observed (*2 slides per animal*)
- Haematoxylin/eosin staining was used for the screening of slides

Results



- The presence of Rickettsia-like organisms (RLO) in the digestive diverticulum of some animals was suspected
- Withering Syndrome (WS) is a wasting disease of abalone, and is caused by the presence of RLO, *Candidatus xenohaliothis californiensis*.

Task 1 - Health status of abalone

Detection of *Candidatus xenohalotis californiensis*

Methodology

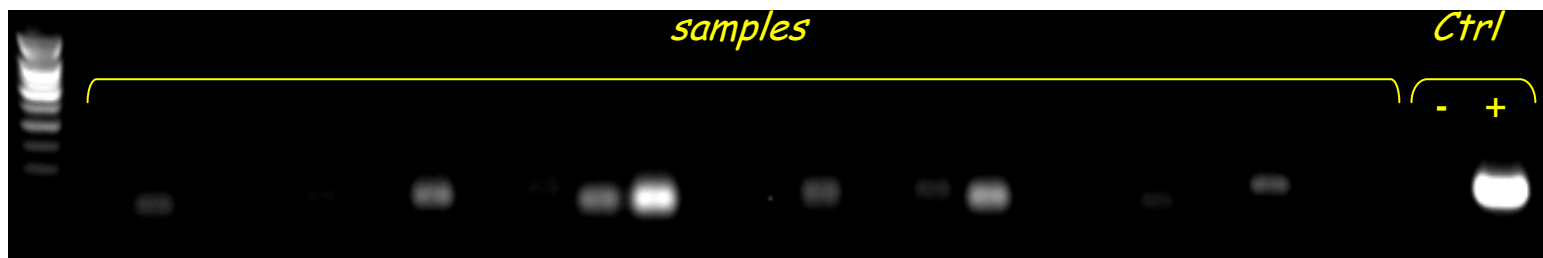
- The PCR protocol recommended by the OIE (World Organisation of Animal Health) code was performed in order to research the presence of *Candidatus xenohalotis californiensis*

Results

- Detection of *Candidatus xenohalotis californiensis* DNA in 50% of animals from both batches (2 and 4 cm)

- Sequence of PCR products corresponds to the expected sequence (*Candidatus xenohalotis californiensis*)

- Nevertheless, other analyses including Feulgen staining and *in situ* hybridisation (ISH) did not allow a visual confirmation of the presence of RLO in samples !



Task 1 - Health status of abalone

Conclusion

- *Candidatus xenohaliotis californiensis* DNA was detected using the PCR technique recommended by the OIE code in abalone cultured in ponds (2cm) or open sea water (4 cm) sampled from FRHAL farm
- This result constitutes **the first evidence of the presence of *Candidatus xenohaliotis californiensis* in abalone on French coasts** and was referred to the French competent authority
- *Candidatus xenohaliotis californiensis* is not a notifiable disease in the European Union and has already been reported in abalone in Ireland and Spain
- Moreover, no abnormal mortality associated to *Candidatus xenohaliotis californiensis* DNA detection was reported in FRHAL farm
- **No other pathogens including *Vibrio harveyi* were detected** in sampled animals

Task 2 - Experimental pathology

Objective : Investigation about the susceptibility of abalone *Haliotis tuberculata* to different pathogens

→ 3 types of pathogens potentially present in the environment of farming abalone were tested

- *Perkinsus olseni*

- Different strains of *Vibrio* spp

- Ostreid herpesvirus 1 (OsHV-1)

Parasite known to infect some mollusc species such as clams, and some species of abalone worldwide (*H. rubra*, *H. laevigata*, *H. scalaris*, *H. cyclobates*)

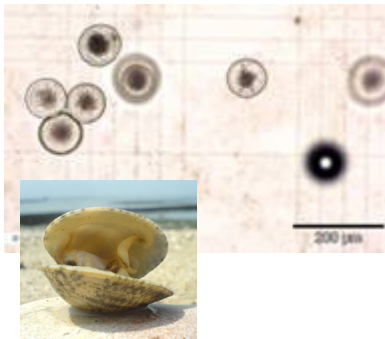
Isolated from moribond oysters during summer mortality outbreaks reported in France, in 2008



Task 2 - Experimental pathology

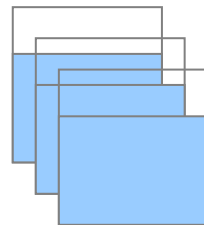
Susceptibility to *Perkinsus olseni*

Methodology

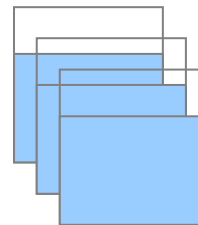


- Cultures of *P. olseni* were prepared from infected clams *Ruditapes decussatus* collected on the French coast (*Marennes-Oleron bay*)
- Experimental infections were performed using balneation of abalone in 5 L of sea water containing 3 billion of *P. olseni* during 2 days at 20°C (*optimal temperature for infection by P. olseni*)
- Sea water was changed every 2 days and mortality was monitored during a period of 5 weeks in absence

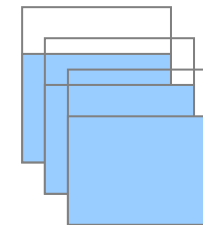
Abalone (30)
without *P. olseni*
Control



Abalone (30)
with *P. olseni*



Clams (healthy) (30)
with *P. olseni*
Control of infection



- After 5 weeks, animals were sacrificed and subjected to thioglycollate analysis (a standard protocol for the detection of *Perkinsus* spp). Animals were also analysed by histology

Task 2 - Experimental pathology

Susceptibility to *Perkinsus olseni*

Results

- No mortality observed in abalone and clams during the course of the experiment

- The thioglycollate analysis indicated that :



40 % of *Ruditapes decussatus* were infected



0 % of *H. tuberculata* were infected

Conclusion

- *Haliotis tuberculata* seems to be not susceptible to *P. olseni* in the tested conditions

Task 2 - Experimental pathology

Susceptibility to different *Vibrio* strains

Methodology



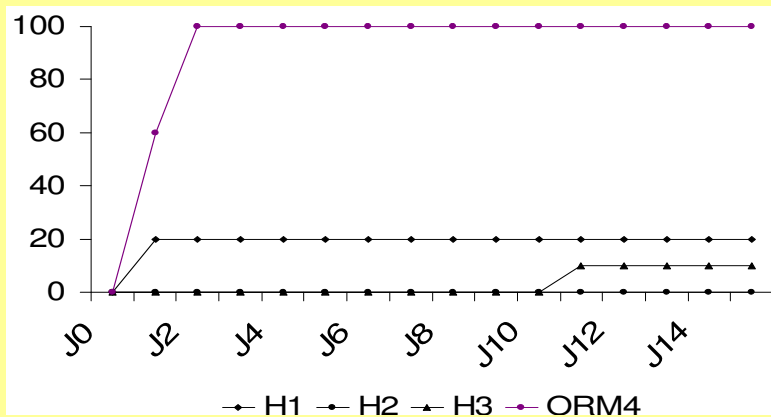
- 6 strains of *Vibrio* spp have been isolated from moribond oysters during summer mortality outbreaks reported in France, in 2008 :
 - 3 strains of *Vibrio alginolyticus*
 - 3 strains of *Vibrio harveyi*
- Pathological experiments were performed by injection of 10^4 bacteria in the foot of animals
- The pathogenic strain *Vibrio harveyi* ORM4 was used as a positive control

Task 2 - Experimental pathology

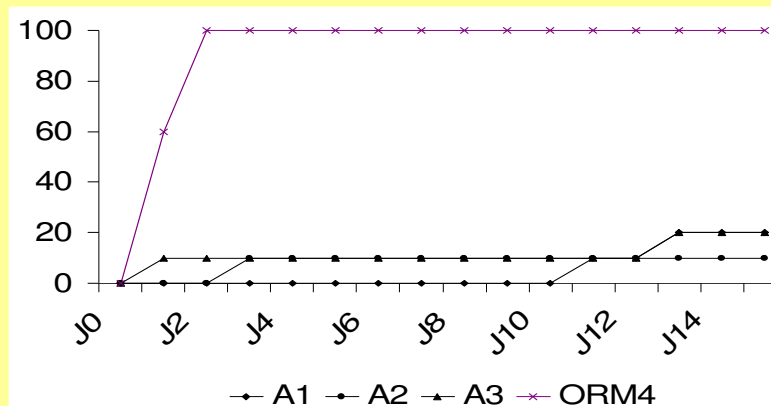
Susceptibility to different *Vibrio* strains

Results

- *Vibrio harveyi*



- *Vibrio alginolyticus*



- Low mortality rates: 20% at J15

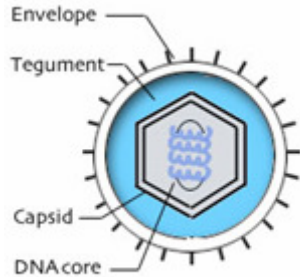
- 100% of mortality for the pathogenic strain *Vibrio harveyi* ORM4 at J2

Conclusion

In comparison to the strain *V. harveyi* ORM4, the different *Vibrio* strains isolated from oysters presented a low pathogenicity to *Haliotis tuberculata*

Task 2 - Experimental pathology

Susceptibility to OsHV-1



- Herpes-like viruses were associated with abalone mortality outbreaks in Australia (*Haliotis laevigata* and *Haliotis rubra*) and in Taiwan (*Haliotis diversicolor*)
- In France, OsHV-1 is implicated in summer mortality outbreaks among Pacific oysters *Crassostrea gigas*.

Question : is OsHV-1 susceptible to present a « risk » for abalone farming ?

Methodology

- 2 types of experimental infections were conducted at 20°C



- Injection : 100µL of a viral suspension of OsHV-1 prepared from experimentally infected oysters were injected in the foot of abalone (4 cm abalone)



- Co-habitation of abalone (1 and 4 cm) with oysters infected by OsHV-1

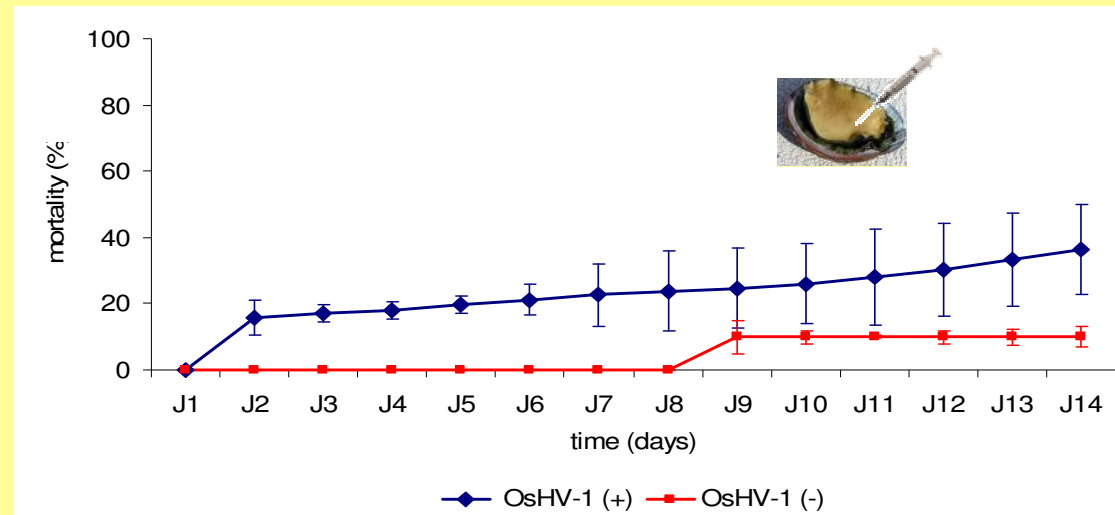
→ more representative of what happens in the natural environment

Task 2 - Experimental pathology

Susceptibility to OsHV-1

Results

By INJECTION



At J14 :

- 40 % of mortality after injection of the viral suspension of OsHV-1
- 10 % of mortality after injection of the control suspension prepared with uninfected oysters

→ Mortality significantly different between animals injected with the OsHV-1 suspension and with the control suspension

→ In contrast, the mortality rate with OsHV-1 appears clearly lower than mortality rates previously described in the literature for herpes-like virus infections reported in Australia and Taiwan and showing around 90% of mortality 3 or 4 days post infection.

Task 2 - Experimental pathology

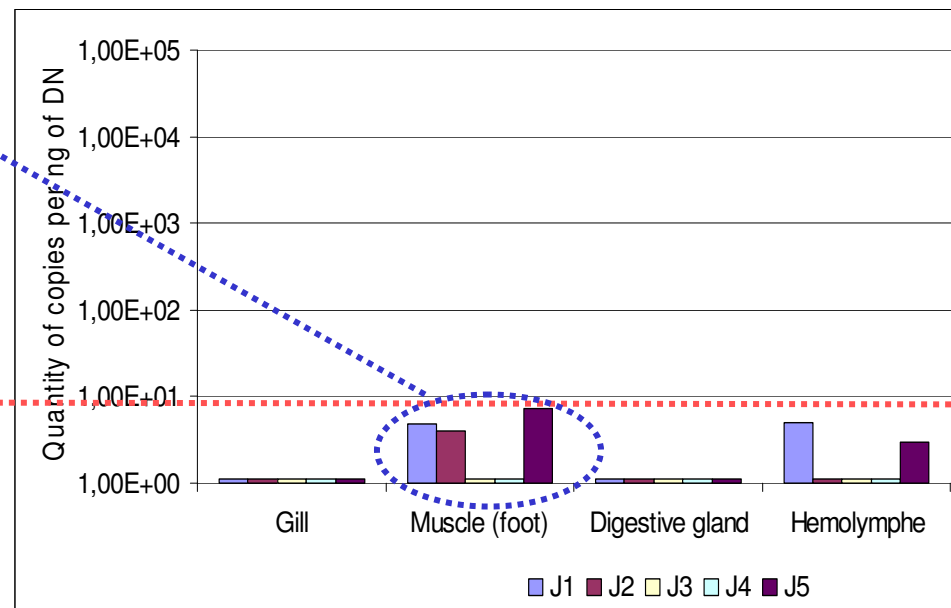
Susceptibility to OsHV-1

Quantification of OsHV-1 by qPCR after injection

- Animals (alive) were daily sacrificed and dissected (gill, foot, digestive gland and haemolymph)
- qPCR (OsHV-1) was performed on DNA extracts

Results

- Viral DNA amounts very low: under 10 copies of OsHV-1 per ng of DNA !
- Principally detected in the foot of animals → injection site of the viral suspension
- In comparison, infected oysters present 10^5 copies of OsHV-1 per ng of DNA at J3 post-infection



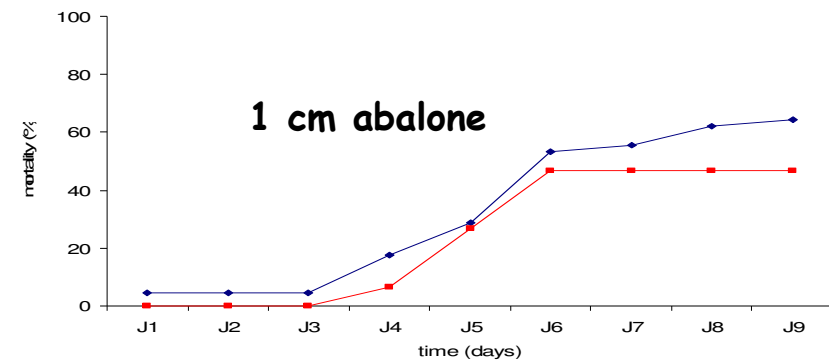
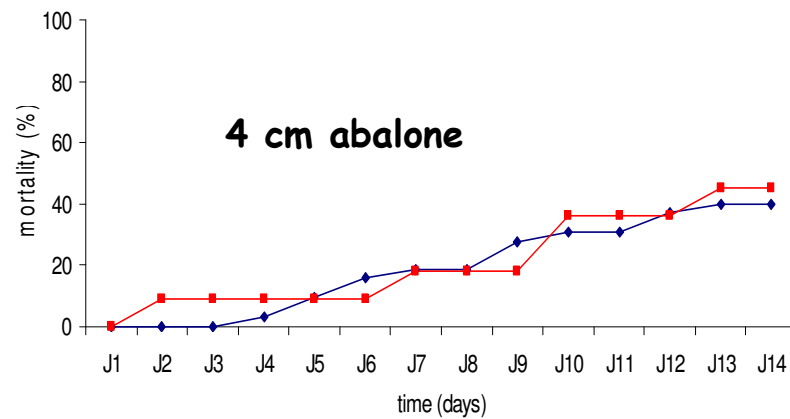
Task 2 - Experimental pathology

Susceptibility to OsHV-1

Results

By Co-HABITATION

40 % of mortality observed at J14 after cohabitation of 1 cm or 4 cm abalone with oysters infected or not with OsHV-1



—◆— with Oysters OsHV-1 (+) —■— with Oysters OsHV-1 (-)

→ No significant mortality in presence of OsHV-1

Task 2 - Experimental pathology

Conclusion

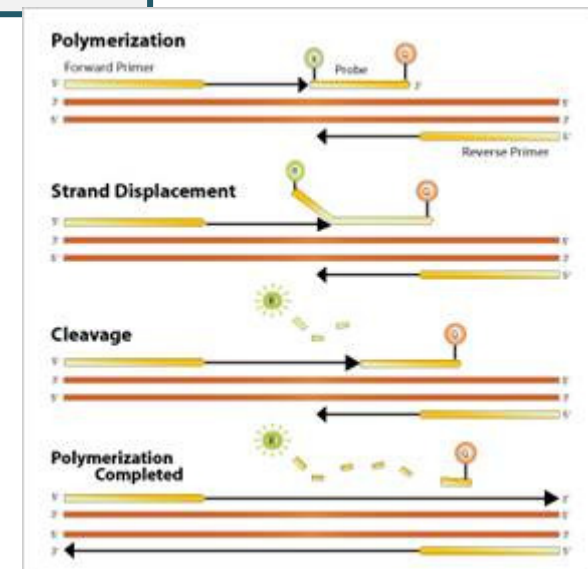
	Pathogens	Mortality	Detection
Experimental pathology	<i>V. harveyi</i> and <i>V. alginolyticus</i> isolated from <i>C. gigas</i>	no	no
	<i>Perkinsus olseni</i>	no	no
	OsHV-1	yes by injection no by balnation	?

Task 4 - Development of specific tools for the diagnosis of *Vibrio harveyi*

- The virulence of the *Vibrio harveyi* strain *ORM4* seems to be associated to the presence of a plasmid, named pVCR1

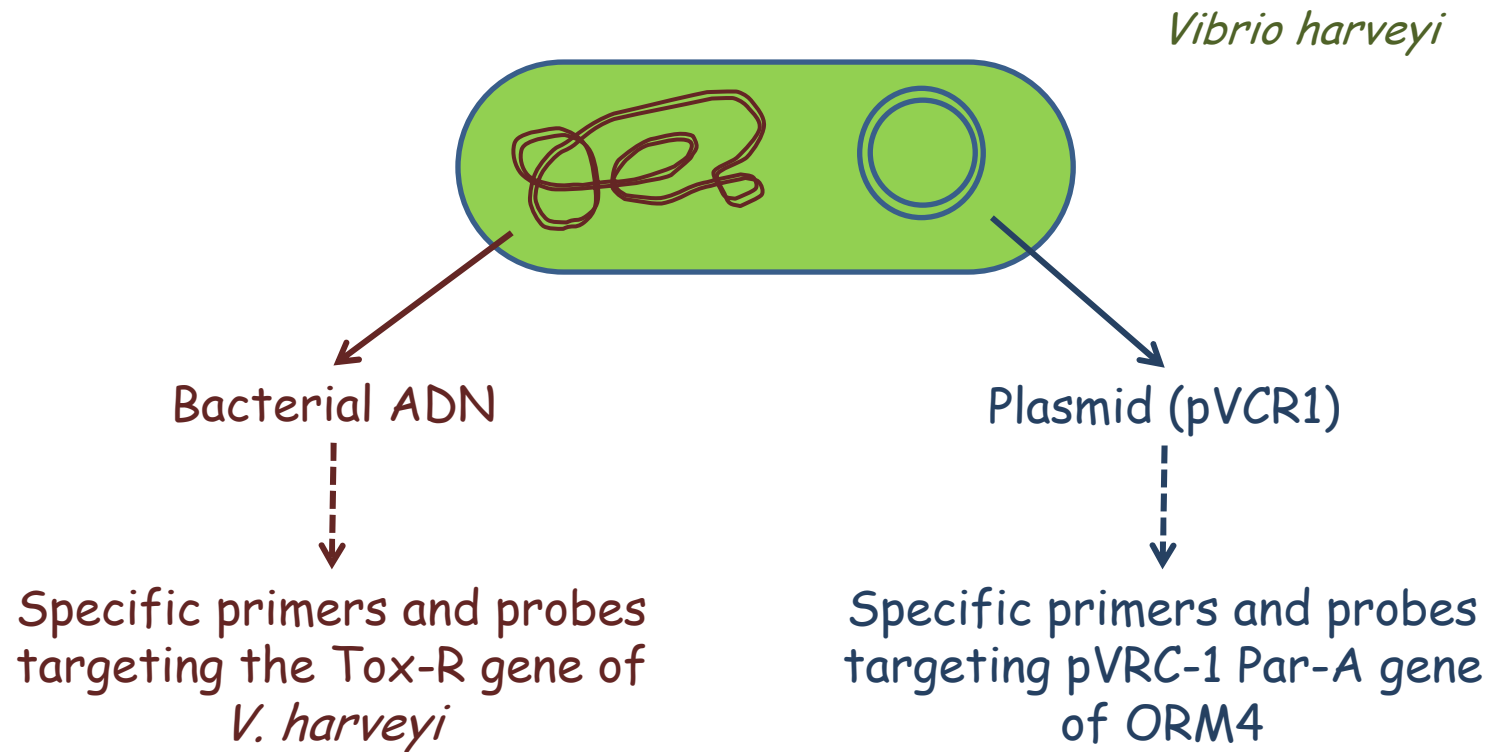
→ Necessity to develop a sensitive and specific detection test of the pathogenic strain *V. harveyi* ORM4

Elaboration of a quantitative PCR protocol using the Taqman® technology



Task 4 - Development of specific tools for the diagnosis of *Vibrio harveyi*

Design of specific primers and probes



Task 4 - Development of specific tools for the diagnosis of *Vibrio harveyi*

Establishment of a standard protocol



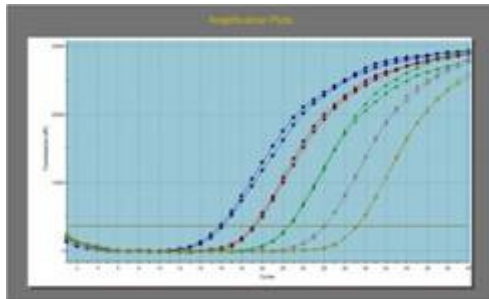
Collection of abalone haemolymph (200 μ L, collection of haemolymph can be performed without sample contamination by bacteria may be present in the external environment).



Total DNA extraction using a commercial kit (Qiagen DNA minikit)



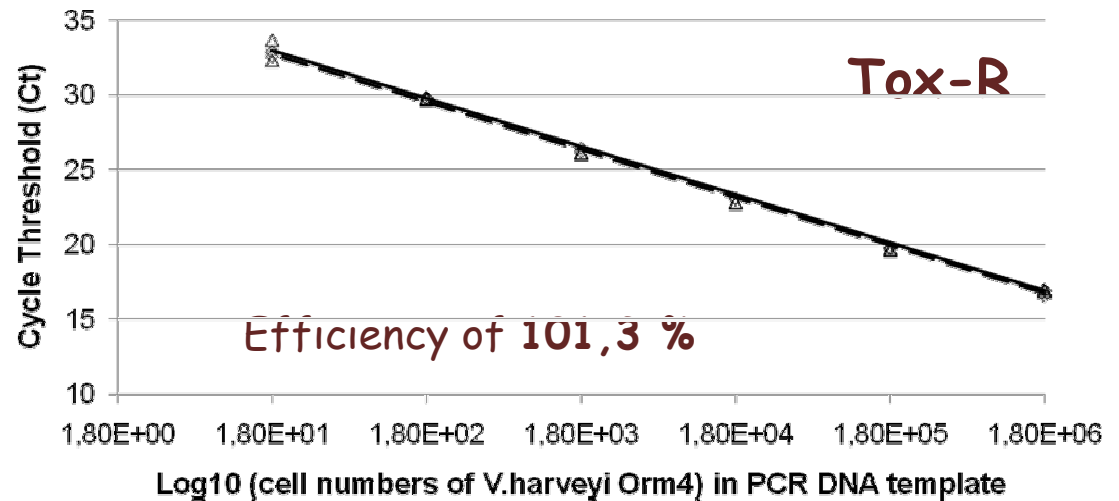
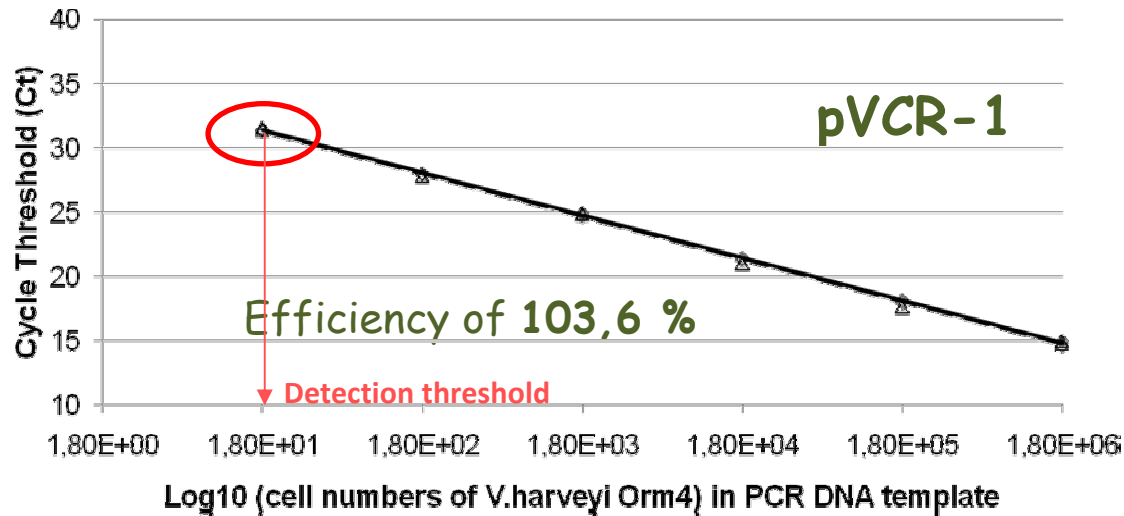
Standardization of the qPCR mix and program



Possibility to detect and quantify the strain *V. harveyi* ORM4 in haemolymph samples

Task 4 - Development of specific tools for the diagnosis of *Vibrio harveyi*

Confirmation of the efficiency of primers and probes



The optimal efficiency for qPCR must be between 95% and 105%

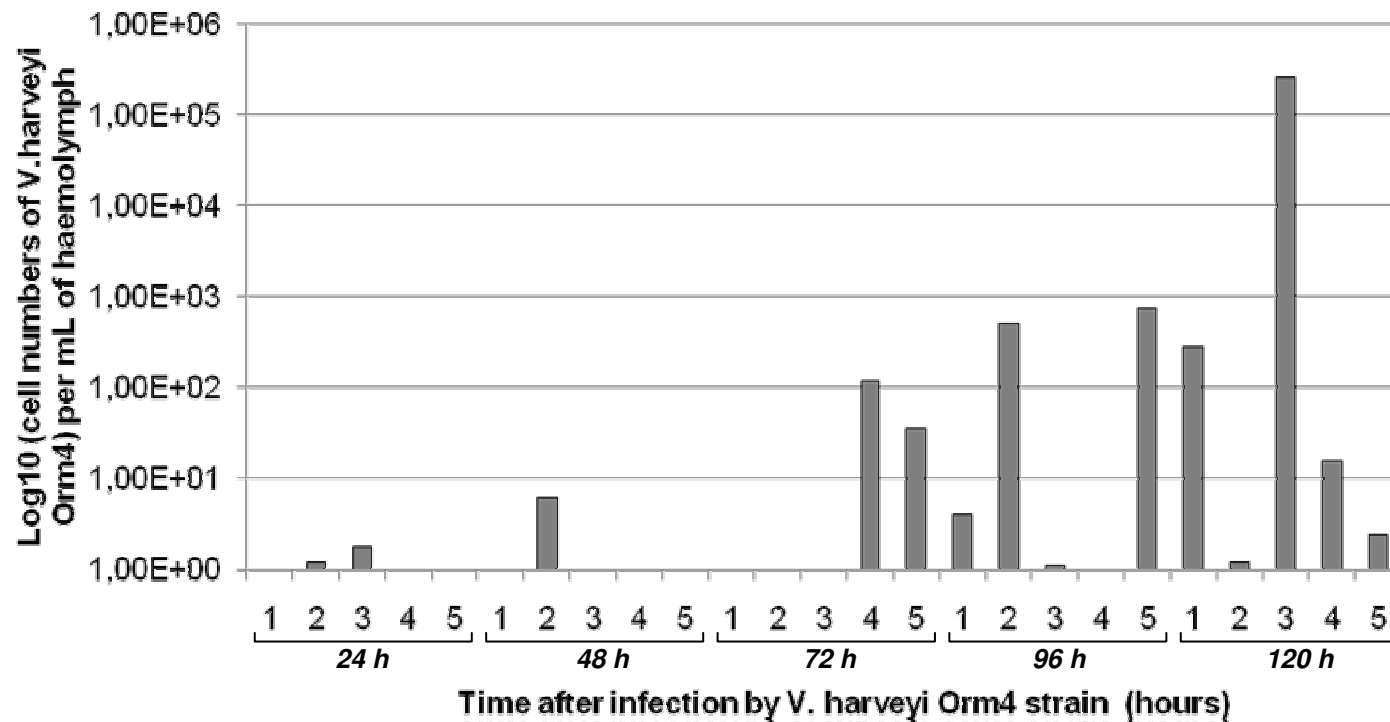
Task 4 - Development of specific tools for the diagnosis of *Vibrio harveyi*

Confirmation of the specificity of primers and probes

Strain	<i>Vibrio</i> species	Real-time PCR results (+/-)	
		<i>tox-R</i> gene	<i>par-A</i> gene
ORM4	<i>V. harveyi</i>	+	+
07/001	<i>V. harveyi</i>	+	-
07/004	<i>V. harveyi</i>	+	-
07/012	<i>V. harveyi</i>	+	-
07/013	<i>V. harveyi</i>	+	-
06/210	<i>V. tubiashii</i>	-	-
DSMZ 17184	<i>V. brasiliensis</i>	-	-
DSMZ 17186	<i>V. rotiferianus</i>	-	-
DSMZ 19137	<i>V. mytili</i>	-	-
CIP 107925	<i>V. corallilyticus</i>	-	-
LMG 11216 T	<i>V. campbelli</i>	-	-
LMG 4409 T	<i>V. alginolyticus</i>	-	-
LMG 2850 T	<i>V. parahaemolyticus</i>	-	-

Task 4 - Development of specific tools for the diagnosis of *Vibrio harveyi*

Vibrio harveyi DNA detection by Q PCR during an experimental infection trial



Task 4 - Development of specific tools for the diagnosis of *Vibrio harveyi*

Conclusion

- Development of the specific diagnostic tool for the detection of bacteria belonging to the *V. harveyi* group (ToxR) and allowing the detection of the pathogenic strain ORM4 (ToxR + pVCR1)
- The use of the Taqman® technology allows to the method a high sensibility and specificity
- Detection threshold of $3 \cdot 10^3$ bacteria per mL of haemolymph
- Feasibility of the qPCR in Simplex (pVCR-1 or Tox-R) or in Multiplex (pPCR-1 and Tox-R) conditions

Thanks for your
attention !

