REPORT ON THE OCCURRENCE OF *Vibrio tapetis* IN KOREAN WATERS: DIAGNOSIS AND ITS IMPACT ON CLAM HEALTH

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Brown ring disease (BRD)

- First observation: in late 1980s from *Ruditapes philippinarum* (Manila clam) in France (Paillard et al., 1989).
- The anomalous deposition of periostracum around the inner shell of the infected organisms.
- The causative agent of BRD was identified as *V. tapetis* (Borrego et al., 1996).
- Decrease in body weight, alteration in biochemical composition (Plana et al., 1996), depressed activities of defense parameters (Allam et al., 2000; Paillard et al., 2004), and mass mortality (Allam et al., 2002).
- Brown ring deposited clam has been often observed on the west coast of Korea for the past few years.
Objectives

1. To identify and characterize BRD syndrome in the Manila clam in Korean water.

2. To understand the effect of BRD on the Manila clam.

3. To understand the current mass mortality of the Manila clam in Korea.
Sample collection
October 2004

Hwangdo
Brown conchiolin deposit

Vibrios (SEM-10,000 X)
**Isolation of V. tapetis**

1. **Extrapallial fluid** from a suspected clam.
2. **Incubation in marine broth** at 18°C for 12 hrs.
3. **Plating on TCBS agar** and incubated at 18°C for 48 hrs.
4. **Isolation of different colony types** and incubation in marine broth at 18°C for 12 hrs.
5. **Isolation of V. tapetis ??**
Identification of *V. tapetis*

- PCR using specific primer pair
- SDS-PAGE
- Amino acid composition
- Cellular fatty acid composition
- 16S-23S rRNA Intergenic Spacer Region (ISR)
- Biochemical tests (API 20E)
- Sequencing of 16S rRNA and phylogenetic analysis

Compared with *V. tapetis* (NCIMB 13622, UK)
For detection of *V. tapetis*

Polymerase Chain Reaction (PCR)

**Expected size: 416 bp**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity</th>
<th>Sequence (5’ - 3’)</th>
<th>Target site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSPVtF</td>
<td><em>V. tapetis</em></td>
<td>CGAGCGGAAACGAGAAGTAG</td>
<td>16S (55-75)</td>
<td>Paillard et al, 2005</td>
</tr>
<tr>
<td>SSPVtR</td>
<td><em>V. tapetis</em></td>
<td>GGATGCACGCTATTAACGTACA</td>
<td>16S (450-472)</td>
<td>Paillard et al, 2005</td>
</tr>
</tbody>
</table>

Genomic DNA  
*N. tapetis*  
(NOIMB 13622)  
*Korean Vibrio sp.*

- 94°C for 2 min
- 94°C for 1 min
- 63°C for 1 min
- 72°C for 1 min
- final extension at 72°C - 5 min
- Stored 4°C

35 cycles
DNA electrophoresis

Vibrio tapetis
NCIMB 13622
Korean Vibrio sp.

416 bp
Cellular fatty acid composition (%)

<table>
<thead>
<tr>
<th>Component</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum in feature 2</td>
<td>0.56%</td>
</tr>
<tr>
<td>12:0</td>
<td>0.61%</td>
</tr>
<tr>
<td>12:0 30H</td>
<td>3.02%</td>
</tr>
<tr>
<td>14:0 ISO</td>
<td>0.54%</td>
</tr>
<tr>
<td>14:0</td>
<td>4.89%</td>
</tr>
<tr>
<td>15:0 ISO</td>
<td>0.53%</td>
</tr>
<tr>
<td>15:0</td>
<td>0.63%</td>
</tr>
<tr>
<td>14:0: ISO 30H</td>
<td>0.33%</td>
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<tr>
<td>Sum in feature 2</td>
<td>6.93%</td>
</tr>
<tr>
<td>16:0 ISO</td>
<td>1.77%</td>
</tr>
<tr>
<td>16:1 w9c</td>
<td>1.56%</td>
</tr>
<tr>
<td>Sum in feature 3</td>
<td>37.51%</td>
</tr>
<tr>
<td>16:0</td>
<td>17.99%</td>
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<tr>
<td>17:0 ISO</td>
<td>0.47%</td>
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<tr>
<td>17:1 w8c</td>
<td>0.73%</td>
</tr>
<tr>
<td>17:0</td>
<td>0.53%</td>
</tr>
<tr>
<td>18:3 w6c</td>
<td>0.43%</td>
</tr>
<tr>
<td>18:1 w7c</td>
<td>17.90%</td>
</tr>
<tr>
<td>18:0</td>
<td>1.32%</td>
</tr>
<tr>
<td>20:0 ISO</td>
<td>0.62%</td>
</tr>
<tr>
<td>20:1 w7c</td>
<td>0.25%</td>
</tr>
</tbody>
</table>

Do not disseminate without author authorization.
Aspartic acid
Glutamic acid
Serine
Histidine
Glycine
Arginine
Alanine
Tyrosine
\@Aminobutyric acid
Valine
Methionine
Phenylalanine
Lysine
Leucine
Isoleucine
Valine
Tyrosine
Arginine
Histidine
Serine
Glutamic acid
Aspartic acid
Korean Vibrio sp.
NCIBM13622
Amino acid composition
Do not disseminate without author authorization
1. Korean *Vibrio* sp.
2. NCIBM13622
<table>
<thead>
<tr>
<th>Biochemical test</th>
<th>Present study</th>
<th>CECTA600</th>
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</thead>
<tbody>
<tr>
<td>β-galactosidase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine dihydrolase</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H₂S production</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tryptophane desaminase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Aceton production</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Gelatinase</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glucose fermentation/oxidation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol fermentation/oxidation</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sorbitol fermentation/oxidation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose fermentation/oxidation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose fermentation/oxidation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Melibiose fermentation/oxidation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amygdalin fermentation/oxidation</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose fermentation/oxidation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inositol fermentation/oxidation</td>
<td>-</td>
<td>-</td>
</tr>
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Sequencing of 16S rRNA of Korean *V*. sp.

Polymerase Chain Reaction (PCR)

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<tbody>
<tr>
<td>1492R</td>
<td><em>Vibrio universal</em></td>
<td>CGGYTACCTTGTTACGAC</td>
<td>16S (1493-1510)</td>
<td>Kalmbach et al, 1990</td>
</tr>
</tbody>
</table>

94°C for 2 min
94°C for 1 min
55°C for 1 min
72°C for 1 min
final extension at 72°C - 5 min
Stored 4°C
Identification of the Vibrio

Sequence homologies were analyzed using the BLAST program for public DNA/protein databases.

The 16S rRNA sequences of Korean Vibrio sp. and other marine Vibrio spp. were aligned with the Clustal W program.

Phylogenetic tree was constructed with neighbor-joining methods using MEGA program version 2.1.
Neighbour-joining phylogenetic tree obtained from 16S rRNA gene sequences

- KOR1
- KOR2
- KOR3

- AY129279
- Y09430

- AJ874387 V. splendidus
- AJ874387 V. vulnificus
- AY862232 V. parahaemolyticus
- AY867727 V. alginolyticus
- AY862014 V. harveyi

Distance scale: 0.005

>99.48% V. tapetis
Comparison of 16S-23S rRNA Intergenic Spacer Region (ISR)

Primers (Kang et al., 2003)

V 16SF: CCGTCACACCATGGGAGTGGG
16S rRNA 3' end

V 23SR: ACTGCCAAGGCATCCACG
23S rRNA 5' end

EF-Taq, 55 °C, 1 min, 30 cycles
Effects of BRD on the Manila clam

Quantification of BRD

infection intensity of BRD according to the infection criteria of Paillard and Maes (1994)

Condition index (CI) of clams

Tissue dry weight (g)/shell dry weight (g)

Correlation test (n=30)

Pearson Correlation:

-0.431 ($P<0.05$)

T-test (n=30)

Lower CI in the BRD infected clams ($P<0.05$)

Negatively correlated with the CI
**V. tapetis** challenge experiment

**Test N=30**

Clams were injected with 100 μl of isolated *V. tapetis* (OD\textsubscript{600}=0.1)

Reared at 20 °C for 14 days

Accumulated mortality (%)

100

**Control N=30**

Clams were injected with 100 μl of sterilized and filtered sea water

Accumulated mortality (%)

20
Conclusions

- **V. tapetis** was isolated from the Manila clam in Korea, and characterized.

- 16S rRNA of Korean *Vibrio* sp. showed that it is the same species as *V. tapetis* in Europe, but difference was observed in 16S-23S rRNA ISR. Further study is needed to confirm its taxonomic position.

- **V. tapetis** infection level was negative correlated with CI. The challenge experiment showed mortality of the clam.

- It is believed that severe disease infection (*V. tapetis* and *Perkinsus olsenii*) is associated with recent mortality of the Manila clam in Korea.
This study was funded by KOSEF of Korea and CNRS of France for scientists exchange program.