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► To cite this version:

Alexandra Rahmani, Maaïke Vercauteren, Katleen Vranckx, Filip Boyen, Adeline Bidault, et al.. MALDI-TOF MS as a promising tool to assess potential virulence of *Vibrio tapetis* isolates. *Aquaculture*, 2021, 530, pp.735729. 10.1016/j.aquaculture.2020.735729 . hal-03083047

HAL Id: hal-03083047

<https://hal.science/hal-03083047>

Submitted on 4 Jan 2021

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1 **MALDI-TOF MS as a promising tool to assess potential virulence of *Vibrio tapetis* isolates**

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43 **ABSTRACT**

44 *Vibrio tapetis*, the etiological agent of Brown Ring Disease, mainly affects the Manila clam *Ruditapes*
45 *philippinarum*. Although this bacterium is mainly known as a clam pathogen, it has been isolated from
46 several fish species. The main aim of the present study was to further explore the variability of 27 *V. tapetis*
47 isolates from bivalves and fish, considering three different aspects; *in vitro* virulence based on the loss of
48 clam hemocyte adhesion properties, detection of the gene *virB4* encoding for an essential component of
49 the Type IV Secretion System, and MALDI-TOF MS characterization based on whole cell extracts. Finally,
50 these approaches were compared and evaluated for their ability to differentiate the potential
51 pathogenicity of the 27 isolates against the Manila clams. Among the 11 *V. tapetis* isolates from the
52 common dab isolated in 2018 in Belgium, only one (2BB) showed intermediate *in vitro* virulence against
53 the Manila clam, and seven carried the *virB4* gene while none of the *V. tapetis* previously isolated from
54 fish in 2003 showed the presence of this particular gene. Finally, the peak protein profiles generated with
55 MALDI-TOF MS analysis from all 27 *V. tapetis* strains showed a clear clustering of clam pathogenic and
56 nonpathogenic isolates suggesting that a new isolate of *V. tapetis* that would cluster within the clam
57 pathogenic isolates could be potentially pathogenic to the Manila clam. Thereupon, MALDI-TOF MS typing
58 allows rapid and cost-efficient identification of *V. tapetis* isolates and can be defined as a complementary
59 method of the traditional qPCR that opens new perspectives to study the virulence of *V. tapetis* isolates
60 but also to perform environmental monitoring in order to prevent outbreaks.

61 **KEYWORDS:** *Vibrio tapetis*, MALDI-TOF MS, *virB4*, hemocyte cytotoxicity assay, Manila clam, fish

62 **ABBREVIATIONS**

63 BRD: Brown ring Disease

64 FSSW: Filtered Sterilized Sea Water

65 HPLC: High performance liquid chromatography

- 66 MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
- 67 MLSA: Multilocus sequence analysis
- 68 RAPD-PCR: Randomly Amplified Polymorphic DNA PCR
- 69 T4SS: Type IV secretion system
- 70 TSA: Tryptic Soy Agar
- 71 TSB: Tryptic Soy Broth
- 72
- 73

74 **1. INTRODUCTION**

75 *Vibrio tapetis* is the etiological agent of Brown Ring Disease (BRD), mainly affecting Manila clam *Ruditapes*
76 *philippinarum*, and responsible for mass mortalities in cultured clams (Maes and Paillard, 1992; Paillard et
77 al., 1994). Clams are amongst the most heavily traded species in the global aquaculture market with Manila
78 clam being the second major cultured bivalve in the world with a yearly production of over 4.4 million ton
79 (FAO, 2018; Smits et al., 2020). BRD is characterized by a brown organic, conchiolin deposit, between the
80 pallial line and the edge of the shell (Paillard and Maes, 1995). The reference strain *V. tapetis* CECT4600,
81 first isolated from a cultured Manila clam exhibiting BRD in France, has been well characterized (Borrego
82 et al., 1996; Paillard and Maes, 1990). Since 1990, *V. tapetis* was identified in BRD of various bivalve hosts
83 such as common cockle *Cerastoderma edule* (Novoa et al., 1998; Paillard and Maes, 1990), rayed artemis
84 *Dosinia exoleta* (Paillard, 2004a), pink clam *Polititapes rhomboïdes* (Paillard, 2004a), grooved carpet shell
85 *R. decussatus* (Maes and Paillard, 1992; Novoa et al., 1998) and *Venerupis aurea* (Maes and Paillard, 1992),
86 This suggests that *V. tapetis* is able to cross species barriers (Paillard, 2016).

87 Since 2003, some studies described the isolation of *V. tapetis* from cultivated or captive held aquatic
88 vertebrates such as corkwing wrasse *Symphodus melops* ((Jensen et al., 2003), Atlantic halibut
89 *Hippoglossus hippoglossus* (Reid et al., 2003), Dover sole *Solea solea* (Declercq et al., 2015), fine flounder
90 *Paralichthys adspersus* and red conger eel *Genypterus chilensis* (Levican et al., 2017). *V. tapetis* was also
91 pinpointed as causative agent of ulcerative skin lesions in the wild-caught common dab *Limanda limanda*
92 (Vercauteren et al., 2019).

93 In the past, *V. tapetis* has been described as a homogenous taxon based on traditional methods such as
94 bio- or serotyping (such as indole production, growth in NaCl and production and utilization of different
95 nutrients) (Allam et al., 1996; Castro et al., 1996; Figueras et al., 1996; Paillard, 2004b). Later studies, using
96 advanced genetic or experimental techniques revealed that this species is more heterogeneous. To date,
97 three distinct subspecies of *V. tapetis* are described, i.e. subsp. *tapetis* (Balboa and Romalde, 2013), subsp.
98 *britannicus* (Balboa and Romalde, 2013) and subsp. *quintayensis* (Levican et al., 2017). However, using

99 genotyping methods, such as Randomly Amplified Polymorphic DNA (RAPD) PCR (Romalde et al., 2002)
100 and multilocus sequence analysis (MLSA) (Gulla et al., 2017) different clusters were defined between *V.*
101 *tapetis* subspecies. This indicates that the phylogeny of *V. tapetis* is not yet completely unraveled.

102 From an epidemical point of view, differences between *V. tapetis* isolates might provide valuable
103 information regarding the diagnosis of BRD outbreaks and detection of virulent isolates. Furthermore,
104 typing of isolates can also help examining the geographical and host distributions supporting a more
105 ecological approach for studying host-pathogen-environment interactions (Paillard, 2016; Rodríguez et al.,
106 2006).

107 Several studies have indeed pointed towards differences between clam pathogenic and non-pathogenic
108 *V. tapetis* strains. *In vivo* infection into the pallial cavity of the clams revealed that none of the tested
109 strains derived from fish are able to induce BRD in manila clam (Bidault et al., 2015; Choquet, 2004; Dias
110 et al., 2018). In addition, these last authors have pointed out that *virB4*, an essential gene coding for
111 nucleoside triphosphatase of the Type IV Secretion System (T4SS), was only present in strains pathogenic
112 to the Manila clam and therefore suggested to be a discriminative tool to differentiate between
113 pathogenic and non-pathogenic strains (Dias et al., 2018). Furthermore, the *virB4* real-time PCR assay
114 offers a method for quantification of *V. tapetis* in the extrapallial fluids of the Manila clam (Bidault et al.,
115 2015).

116 The main aim of the present study was to further explore the variability between the 27 *V. tapetis* isolates
117 from bivalves and fish, considering three different aspects; *in vitro* virulence based on the loss of hemocyte
118 adhesion properties after contact with *V. tapetis* (Choquet et al., 2003), detection of the *virB4* gene using
119 the TaqMan qPCR (Bidault et al., 2015), and protein-based matrix-assisted laser desorption/ionization
120 time-of-flight mass spectrometry (MALDI-TOF MS) characterization. Finally, these approaches were
121 compared and evaluated for their ability to discriminate the potential virulence of the 27 isolates against
122 the Manila clams.

123 2. MATERIALS AND METHODS

124 **2.1. Bacterial isolates and cultivation**

125 The *V. tapetis* isolates used in this study were isolated from nine different host species (five bivalves and
126 four fish species) in various countries between 1988 and 2017. Table 1 summarizes the information of all
127 isolates with the year, place and host of first isolation and a synthesis of the existing knowledge about
128 their virulence. After isolation, all isolates were frozen at -80 °C for further analysis

129 For the cytotoxicity bioassay, only the isolates derived from common dab and the CECT4600 reference
130 strain were used. All isolates were cultivated on Tryptic Soy Agar (TSA; Difco™) supplemented with 1.5 %
131 NaCl and were allowed to grow for two days at 16 ± 1 °C (fish isolates) or 18 ± 1 °C (reference clam isolates).

132 For the *virB4* gene detection only common dab isolates were used. All isolates were cultured in TSA
133 supplemented with 1.6 % NaCl for PCR and Tryptic Soy Broth (TSB; Difco™) supplemented with 1.6 % NaCl
134 for qPCR. For MALDI-TOF analysis, all 27 isolates were cultivated in triplicate (biological replicates,
135 Supplementary file 1) on TSA supplemented with 1.5 % NaCl. Isolates were grown for minimally two days
136 at 16 ± 1 °C (fish isolates) or 18 ± 1 °C (clam isolates). We chose to use the optimal or common
137 temperatures for the strains to ensure optimal growth of the isolates.

138 **2.2. In vitro hemocyte cytotoxicity bioassay**

139 Virulence of *V. tapetis* isolates derived from common dab was tested using the standardized *in vitro*
140 hemocyte cytotoxicity bioassay (Choquet et al., 2003). This test is based on the ability of *V. tapetis* virulent
141 strains to induce a rounding phenotype on infected hemocytes, increasing the number of non-adherent as
142 compared to the negative control. *V. tapetis* induces during its phagocytosis, a process of cytoskeletal
143 inhibition and cell rounding. Thus, this adhesion test provides not only morphological but also functional
144 characterization. The type strain CECT4600 was used as the positive control, and filter-sterilized seawater
145 (FSSW) as negative control. Animals used in this study were Manila clams (4 cm Manila clam, February
146 2018) from the SATMAR shellfish aquaculture site in Landeda (Finistère, France). Clams were allowed to
147 acclimate in oxygenated seawater at 14°C for 14 days. Hemolymph was harvested from the adductor
148 muscle, pooled after quality check and the hemocytes were enumerated using a Malassez counting grid.

149 100 µL of hemolymph ($5 \cdot 10^5$ hemocyte/mL) was added in 24-well plates and kept for a few minutes to let
150 hemocytes adhere to the bottom of the plate. Then, 100 µl of bacterial suspension prepared with FSSW,
151 was added at a bacteria/hemocyte ratio of 25/1. Hemocyte exposure to each of the bacterial isolates was
152 performed in triplicate. In negative control samples, 100 µl of FSSW was added to the hemolymph. After
153 3 h of incubation, the number of non-adherent hemocytes was measured (used as a proxy of *V. tapetis*
154 cytotoxicity) using flow cytometry after addition of 4 µl of SYBR–Green solution in DMSO (nucleic acid gel
155 stain, dilution 1 : 10 000, Life Technology, USA) as already described (Choquet et al., 2003). A ratio was
156 calculated by dividing the number of non-adherent hemocytes in samples exposed to bacteria by the
157 number of non-adherent cells in negative controls. Statistical analyses were performed using a pairwise
158 Student test to determine significant differences in non-adherent cell ratios between bacterial suspensions
159 and both positive and negative control samples as already performed by Choquet et al. (2003).

160 **2.3. *VirB4* gene detection**

161 A PCR assay was performed with common dab isolates, in which a fragment of 173 bp of the *virB4* gene
162 was amplified using primers 170513 (5'-TTAAAAGTGGCGGAGGAATG-3') and 170514 (5'-
163 AAGCTCTGCATCGGTTAGGA-3') and GoTAQ polymerase. Subsequently, a Taqman real-time qPCR
164 quantification was performed in triplicate according to the standardized method previously described
165 (Bidault et al., 2015). The positive control used for both of these experiments was the strain *V. tapetis*
166 CECT4600.

167 **2.4. MALDI-TOF MS characterization**

168 An ethanol formic acid extraction was performed, based on MALDI Biotyper protocol (Bruker Daltonics,
169 Bremen Germany). Briefly, bacterial cultures were suspended in nuclease-free water aliquoted in 1.5 mL
170 Eppendorf tubes. Ethanol (70 %) dissolved in high performance liquid chromatography (HPLC) grade water
171 was added to the suspension and tubes were centrifuged twice ($20\,000 \times g$, 2 min) upon which the ethanol
172 was removed. Thereafter, 20 µL of 70% formic acid (in HPLC grade water) was added to the pellet. To finish
173 the extraction, 20 µL of acetonitrile was added. One µL of each extract was spotted eight-fold (technical

174 replicates) on a MALDI target plate (Bruker Daltonics, Bremen, Germany), air dried and covered with 1 μ L
175 of alpha-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Bremen, Germany). All spots were
176 processed in triplicate (technical replicates, Supplementary file 1) with an Autoflex III Smartbeam MALDI-
177 TOF MS, recording masses ranging from 2 000 to 20 000 Da using standard settings (flexControl 1.4, version
178 3.4, Bruker Daltonic, Bremen, Germany). The obtained raw spectra were imported in BioNumerics 7.6.3
179 (Applied Maths NV, Sint-Martens-Latem, Belgium) for data analysis. Preprocessing of the data was
180 performed according to (Giacometti et al., 2018). After preprocessing, peaks were detected using the
181 continuous wavelet transform method with a signal-to-noise threshold of two. The spectra were
182 summarized in an average spectrum per biological replicate (Supplementary file 1) and all replicates with
183 less than 95% similarity to this summary spectrum were removed from the analysis. Of *V. tapetis* 2BB,
184 RD0705 and RP2.3, only two biological replicates were implemented in the analyses due to inconsistencies
185 in the data or low similarity (< 50%) with other replicates of the same isolate. The resulting summary
186 spectra were used to construct an UPGMA dendrogram using a Pearson similarity coefficient.

187 **3. RESULTS AND DISCUSSION**

188 Infectious diseases remain one of the main limiting factors in the aquaculture of clams, therefore
189 increasing the value of research on the variability of those infectious agents (Smits et al., 2020). The wide
190 host (at least fourteen different host species) and geographic distribution (at least seven different
191 countries) of *V. tapetis* and linked questions on possible variability of isolates have been discussed in
192 previous research with a deep interest in finding a discriminative test for assessing pathogenicity of *V.*
193 *tapetis* isolates against clams (Bidault et al., 2015). Furthermore, the discovery of new isolates from skin
194 ulcerations in the common dab (Vercauteren et al., 2018) might urge for further exploration of pathogenic
195 markers using available techniques.

196 **3.1. Most of the *V. tapetis* isolates from the common dab are unable to induce hemocyte toxicity.**

197 To gain insight into the pathogenicity of *V. tapetis* to the Manila clam, an *in vitro* cytotoxicity assay was
198 developed based on the cell-rounding and subsequent loss of adherence of hemocytes following exposure

199 to *V. tapetis* (Choquet et al., 2003). This bioassay was previously performed for many *V. tapetis* strains
200 pathogenic to bivalves (Table 1), whereby strains IS9 and CECT4600 induced the highest loss of adhesion
201 of the hemocytes (i.e. *in vitro* cytotoxicity). Most of the other strains from clams displayed intermediate
202 cytotoxicity and only a few showed no cytotoxicity (Table 1). Remarkably, the GDE and GTR-I strains, both
203 isolated from bivalves but not from the Manila clam, showed no cytotoxicity *in vitro*. Complete genome
204 analyses of these two strains revealed strong differences with clam isolates (Dias et al., 2018) and these
205 two strains were genetically closer to the LP2 strain derived from corkwing wrasse. This results further
206 substantiates the observed difference in cytotoxicity towards Manila clam hemocytes (Dias et al., 2018).

207 The common dab isolates, 2BG, 2AE, 2AC and 2BW tested in this study, showed non-adherent cells ratios
208 after bacterial exposure fluctuating around 1. The 2BU isolate displayed a ratio of 0.85 ± 0.14 and remained
209 clearly below 1 in all replicates. The ratios found with isolates 2BC, 2BT, 2AU, and 2BA ranged between 1
210 and 1.5. All isolates showed similar ratios with the FSSW control, therefore indicating negative cytotoxicity
211 (i.e. causing no additional loss of adherence of hemocytes) ($pvalue > 0.05$; Fig 1). The isolate 2BB showed
212 the highest cytotoxic activity with an average ratio of 2.27 ± 0.26 (Fig 1). The cytotoxicity of 2BB was
213 evaluated to be intermediate since the amount of non-adherent hemocytes was significantly higher as
214 compared to the FSSW control ($pvalue: 0.0005$) but was intermediate considering the positive control
215 strain ($pvalue: 0.31$) These results are consistent with previous ones obtained with strains isolated from
216 fish (e.g. LP2 and HH6087) which mostly showed negative cytotoxicity towards clam hemocytes (Dias et
217 al., 2018).

218 Comparison between different *in vitro* cytotoxicity bioassays is complicated since the non-adherent
219 hemocyte ratio might vary depending on the susceptibility of hemolymph to *V. tapetis*. This is illustrated
220 with the strain LP2, which showed intermediate cytotoxicity in Choquet et al. (2003) and negative
221 cytotoxicity in Dias et al. (2018). In the present study, this bias risk was reduced, by using one pool of
222 hemocytes and using a standardized analysis of the data comparing the results to both a positive
223 (CECT4600) and negative control (FSSW), as already described in Choquet et al. (2003).

224 **3.2. First detection of the *virB4* gene in non-cytotoxic fish isolates**

225 Recently, a rapid and accurate Taqman real-time PCR assay for detection and quantification of *V. tapetis*
226 in extrapallial fluids of the Manila clam has been developed, based on the presence of the *virB4* gene,
227 which encodes a component of the T4SS (Bidault et al., 2015). Since the T4SS has been described to be
228 essential for virulence in other pathogenic species such as *Helicobacter pylori* and *Legionella pneumophila*
229 (Voth et al., 2012) ,this PCR analysis was suggested as a possible screening tool to differentiate between
230 clam pathogenic and non-pathogenic isolates (Dias et al., 2018). Among 17 fully sequenced *V. tapetis*
231 genomes, in a previous study, the T4SS genes cluster was identified only in the genomes of isolates virulent
232 to the Manila clam (based on *in vivo* assays), thus proving that these strains do not only carry the *virB4*
233 gene but the entire T4SS gene cluster and also suggesting a role for this T4SS system in *V. tapetis*
234 pathogenicity towards clams (Dias et al., 2018). It should be noted that FPC1121, an isolate from Manila
235 clam in Japan, does show *in vivo* virulence towards Manila clam, even though the *virB4* gene is not present.
236 Surprisingly, in the isolates from common dab, the *virB4* gene was found to be present in seven out of the
237 11 isolates (2BM, 2AU, 2BA, 2BT, 2BU, 2AC and 2BG). This study is therefore the first to provide evidence
238 that *V. tapetis* isolated from fish can carry the *virB4* gene, in contrast with previously reported results (Dias
239 et al., 2018). It needs however to be elucidated if these *virB4* positive isolates carry the entire cluster
240 coding for T4SS and/or are able to induce BRD during *in vivo* experiments. Therefore, it seems that it was
241 not the only gene that determines virulence, as expected.

242

243 **3.3. MALDI-TOF MS analysis reveals 3 clusters of *V. tapetis* isolates**

244 All 27 *V. tapetis* isolates included in this study were analyzed using the MALDI-TOF MS method. This
245 method allows sensitive and rapid identification of microorganisms and is now widely used in different
246 fields such as clinical microbiology, epidemiological studies and water or food borne pathogens (Maier et
247 al., 2006; Singhal et al., 2015). The technique is broadly used to identify microorganisms at the species
248 level, but has recently been shown valuable for strain typing (Sandrin et al., 2013).

249 The main peaks generated by the MALDI-TOF MS were found between 2000 and 7500 Da. Based on the
250 peak profiles, the constructed dendrogram (Fig 2) of the investigated spectra revealed some clearly
251 delineated clusters. In total three distinct clusters (named A, B and C) were defined, based on differences
252 in protein profiles (Fig 2). All strains that were derived from BRD outbreaks in cultured Manila clam
253 between 1988 - 1996 clustered together in cluster B with only a 29.6 % similarity with other isolates. The
254 strains derived from the common cockle (IS9) and from the carpet shell clam, *R. decussatus* (RD0705) were
255 also included in this cluster. All these strains were collected during the BRD emergence in Europe, which
256 might explain the limited variability between the isolates. The two strains that were isolated from the
257 rayed artemis (GDE) and pink clam (GTR-I) were clustered together with isolates from fish, in cluster A.
258 The latter cluster could be further divided in four sub-clusters (Fig 2). Interestingly, one isolate (2BW)
259 demonstrated different protein profiles and was included in a separate cluster C. This bacterium was
260 isolated as a co-culture with *Pseudoalteromonas* sp. and *Psychrobacter submarinus* from a skin ulcer in dab.
261 *In vitro* assays demonstrated the absence of the *virB4* gene and hemocyte cytotoxicity. Therefore, it is
262 possible that this isolate represents a non-pathogenic strain for clams and/or dab. The virulence of this
263 isolate should be characterized towards clams and fish to elucidate this clustering. Increasing the number
264 of isolates (from different hosts and geographic origin) would corroborate our results and fortify the
265 importance of MALDI-TOF MS analysis for differentiate between clam pathogenic and non-pathogenic *V.*
266 *tapetis* isolates.

267 The MALDI-TOF MS clustering in the present study showed some similarities with previously reported data
268 based on genome analyses (Dias et al., 2018). The latter have demonstrated that the two strains isolated
269 from the rayed artemis (GDE) and pink clam (GTR-I) clustered together with LP2 isolated from corkwing
270 wrasse, and were genetically distant from *V. tapetis* strains isolated from clams.

271 FPC1121 is the only clam pathogenic strain that was clustered together with fish isolates, based on the
272 MALDI-TOF MS profiling. FPC1121 was isolated from cultivated Manila clam in Japan (Table 1, Matsuyama
273 et al., 2010). The original report clearly described the brown deposit and mass mortalities of Manila clam

274 due to this strain, which was confirmed by *in vivo* virulence assays (Matsuyama et al., 2010). This clustering
275 of FPC1121 is an interesting result; detailed genetic analysis would be interesting to explore the
276 phylogenetic position of this strain and the linked genetic differences.

277 **3.4. Value of the assays to differentiate isolates according to their host-species (Fig 3)**

278 *In vivo* virulence tests are necessary to determine the ability for an isolate to induce BRD. However, several
279 tests have been developed to characterize the *in vitro* virulence regarding Manila clam.

280 The *in vitro* cytotoxicity assay has been used as a tool to evaluate the pathogenicity of *V. tapetis* strains
281 for clams (Dias et al., 2018; Bidault et al., 2015). This assay is based on the correlation between the
282 cytotoxic activity of bacteria to clam hemocytes and the *in vivo* pathogenicity. As demonstrated in Table
283 1, such a correlation was not found for strain RD0705. Indeed, this strain causes no hemocytes cytotoxicity
284 *in vitro* although it can cause BRD *in vivo* in the Manila clam (Table 1) (Dias et al., 2018; Novoa et al., 1998).
285 Since RD0705 was isolated from another clam species (i.e. the grooved carpet shell), it is tempting to
286 speculate that this inconsistency might be related to host specificity. However, it could also suggest that
287 cytotoxicity towards hemocytes is not the only virulence factor involved in the development of BRD. Beside
288 this exception, it should be recalled that the analysis of the virulence profiles of many *V. tapetis* strains
289 isolated from clam revealed a good correlation between the cytotoxic activity to clam hemocytes and the
290 *in vivo* pathogenicity.

291 Another assay commonly used to detect *V. tapetis* isolates pathogenic to clams is the search for the *virB4*
292 gene encoding part of the T4SS (Dias et al., 2018). In our study, we have demonstrated for the first time
293 that the *virB4* gene can also be present in fish isolates. Since these isolates did not display *in vitro* virulence
294 to clam hemocytes and they clustered separately based on their protein profile, it could be possible that
295 they are not pathogenic for clams. *In vivo* studies should be performed to confirm the pathogenicity
296 towards clams and if so, this might question the use of *virB4* detection as a pertinent marker for
297 pathogenicity to clams.

298 The MALDI-TOF dendrogram showed a good clustering of *V. tapetis* from different origins. However, this
299 clustering was not correlated with the presence of the *virB4* gene. In fact, the clustering was more
300 correlated with the host species from which they were isolated. Based on the results of the different assays
301 in the present study, it could be hypothesized that MALDI-TOF clustering could differentiate between clam
302 pathogenic and nonpathogenic isolates.

303 ***3.5. Implications in the context of virulent strain detection***

304 Based on these results, MALDI-TOF MS analysis seems to be a promising tool to indirectly evaluate the
305 pathogenicity of a *V. tapetis* isolate towards the Manila clam. Nevertheless, although the presented
306 MALDI-TOF MS isolate typing might be a rapid, cost-effective and powerful tool in identifying *V. tapetis*
307 isolates, the Taqman real-time qPCR remains necessary to quantify the load of *V. tapetis* during infection.
308 Although some inconsistencies exist, the *in vitro* assay is also believed to give a good indication of virulence
309 towards Manila clam in some cases.

310 Regarding the value of each test described in this study to determine *V. tapetis* isolates pathogenicity to
311 the Manila clam, we can consider the MALDI-TOF MS as a new promising screening tool which can be
312 complementary to the tools already used, increasing reliability of the screening.

313 **4. CONCLUSION**

314 In conclusion, the discovery of new *V. tapetis* isolates derived from common dab was linked with the need
315 for a further exploration of variability of *V. tapetis* isolates. The currently used assays (toxicity to
316 hemocytes and search of the *virB4* gene), have previously been shown to be interesting to differentiate
317 between *V. tapetis* pathogenic and non-pathogenic clam isolates. Nevertheless, in this study, *V. tapetis*
318 isolates from dab showed inconsistencies with results of previously pinpointed techniques questioning
319 their discriminative power. In contrast, the MALDI-TOF MS analysis proved to be a promising tool with the
320 possible ability to differentiate between pathogenic and non-pathogenic clam isolates. This can be used
321 as a complementary discriminative test for virulence of clam isolates.

322 This approach allows rapid and cost-efficient identification of *V. tapetis* species and opens new
323 perspectives to study the virulence of *V. tapetis* isolates but also to perform environmental monitoring in
324 order to prevent outbreaks.

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425

426

427 **ACKNOWLEDGEMENTS**

428 We warmly thank Annelies M. Declercq that provides us the first isolates of *V. tapetis* at the beginning of
429 this collaboration. We would like to acknowledge Serge Verbanck for the great work with the MALDI-TOF.

430 **AUTHORS CONTRIBUTIONS**

431 The collaboration has been initiated by AD, KC, CP and VP. *In vitro* virulence assays were performed by MV
432 and AR. *virB4* detection was performed by MV, AR and AB. MALDI TOF MS experiments and analyses were
433 performed by MV, FB and KV. The article was written by MV, AR, CP, VP, KC and AD.

434 **CONFLICTS OF INTEREST**

435 Alexandra Rahmani declares that she has no conflict of interest.

436 Maaïke Vercauteren declares that she has no conflict of interest.

437 Katleen Vranckx is an employee of Applied Maths NV (bioMérieux SA).

438 Filip Boyen declares that he has no conflict of interest.

439 Adeline Bidault declares that she has no conflict of interest.

440 Vianney Pichereau declares that he has no conflict of interest.

441 Annemie Decostere declares that she has no conflict of interest.

442 Christine Paillard declares that she has no conflict of interest.

443 Koen Chiers declares that he has no conflict of interest.

444 **FUNDING**

445 The research was funded by the European Fisheries Fund (EVF – project VIS/15/A03/DIV), the Flemish
446 Government and the Research Foundation – Flanders (FWO). This work makes use of the resources,
447 facilities and/or services provided by UGent and Flanders Marine Institute as part of the Belgian

448 contribution to EMBRC-ERIC. This project received grants from the H2020 European project “VIVALDI”
449 (grant agreement N°678589). This work was also supported by the “Université de Bretagne Occidentale”
450 (UBO, France), and the “investment for the future” programs LabexMER (ANR-10-LABX-19) and ISblue
451 (ANR-17-EURE-0015). The MALDI-TOF mass spectrometer was financed by the Research Foundation
452 Flanders (FWO-Vlaanderen) as Hercules project G0H2516N (AUGE/15/05). The funding bodies had no role
453 in the study design, data collection, analysis or the writing process of the manuscript.

454 **Compliance with Ethical Standards**

455 All applicable international, national, and/or institutional guidelines for the care and use of animals were
456 followed.

457 This article does not contain any studies with human participants performed by any of the authors.

458 **TABLES**

459 **Table 1:** Information on the *V. tapetis* isolates of clams, other bivalves and fish with host species, common name of the host species, description of the health
460 of the host species, year and location of isolation and a reference for each isolate. Furthermore, a synthesis is provided of the existing knowledge on (1) *in*
461 *vivo* virulence, estimating the possibility of the isolate to cause Brown Ring Disease in Manila clam following experimental infection. The isolate indicated with
462 an asterisk is able to cause Brown Ring Disease in pink clam but not in Manila clam; (2) *in vitro* cytotoxicity against hemocytes of the Manila clam ((-): no
463 cytotoxicity, (±) intermediate cytotoxicity, (+):cytotoxicity. a: negative in Dias et al, 2018 and intermediate in Choquet, 2004. b: negative in Dias et al, 2018
464 and intermediate in Choquet et al, 2003 And (3) presence of the *virB4* gene from Bidault et al., 2015; (+) : presence, (-): absence. References for the virulence
465 analyses are: Bidault et al., 2015; Dias et al., 2018; Novoa et al, 1998; Choquet et al, 2004; Matsuyama et al., 2010. Nd: Not determined; *: Determined in this
466 study.

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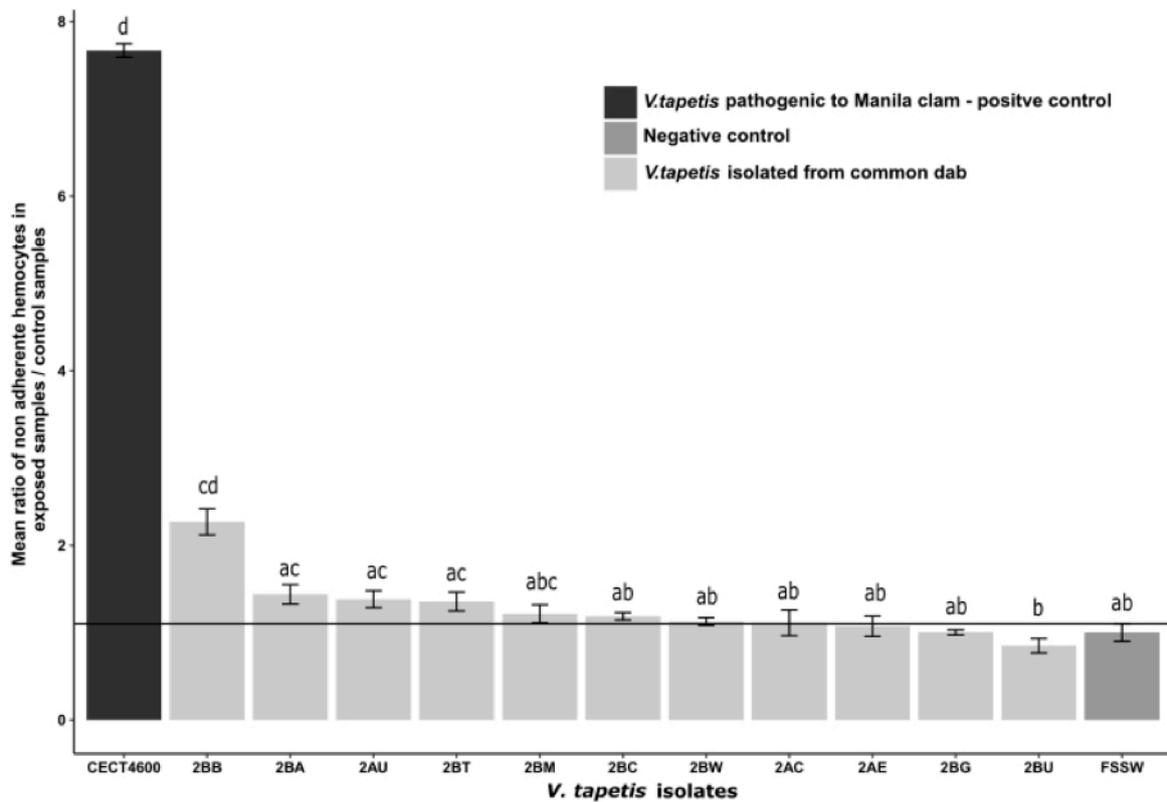
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Isolates	Host species	Common name	Isolated from	Year	Location	Isolate reference	<i>In vivo</i> virulence (1)	<i>In vitro</i> cytotoxicity (2)	<i>virB4</i> gene detection (3)
CLAMS									
RD0705	<i>Ruditapes decussatus</i>	Grooved carpet shell	Animals with BRD signs	1992	Spain, Galice	(Novoa et al., 1998)	+	-	+
CECT4600	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996; Paillard and Maes, 1995)	+	+	+
FPC1121	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	2008	Japan	(Matsuyama et al., 2010)	+	Nd	-
IS1 (VP1)	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1988	France, Landeda	(Paillard and Maes, 1990)	+	±	+
IS5	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1991	France, Landeda	(Borrego et al., 1996)	+	±	+
P16B	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1995	France, Golfe du Morbihan	(Allam et al., 2002)	+	±	+
RP11.2	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	±	+
RP2.3.	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	±	+
RP8.17	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	± ^{-a}	+
RP9.7	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	±	+
UK6	<i>Ruditapes philippinarum</i>	Manila clam	Animals with BRD signs	1996	Great Britain	(Allam et al., 2000)	+	±	+
OTHER BIVALVES									
IS9	<i>Cerastoderma edule</i>	Common cockle	Healthy animals	1990	France, Quiberon	(Borrego et al., 1996)	+	+	+
GTR-I	<i>Polititapes rhomboïdes</i>	Pink clam	Animals with BRD signs	2008	France, Glénan	(Dias et al., 2018)	-*	-	-
GDE	<i>Dosinia exoleta</i>	Rayed artemis	Animals with BRD signs	2003	France, Glénan	(Dias et al., 2018)	-	-	-
FISH									

LP2	<i>Symphodus melops</i>	Corkwing wrasse	Kidney of fish suffering vibriosis	1999	Norway, Bergen	(Jensen et al., 2003)	-	±/. ^b	-
HH6087 (CECT8161)	<i>Hipoglossus hipoglossus</i>	Atlantic halibut	Kidney of moribund fish	2002	Great Britain, Glasgow	(Reid et al., 2003)	-	-	-
2AC	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2AE	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	-*
2AU	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BA	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BB	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	±*	-*
2BC	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	-*
2BG	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BM	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BT	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BU	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	+*
2BW	<i>Limanda limanda</i>	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	-*	-*

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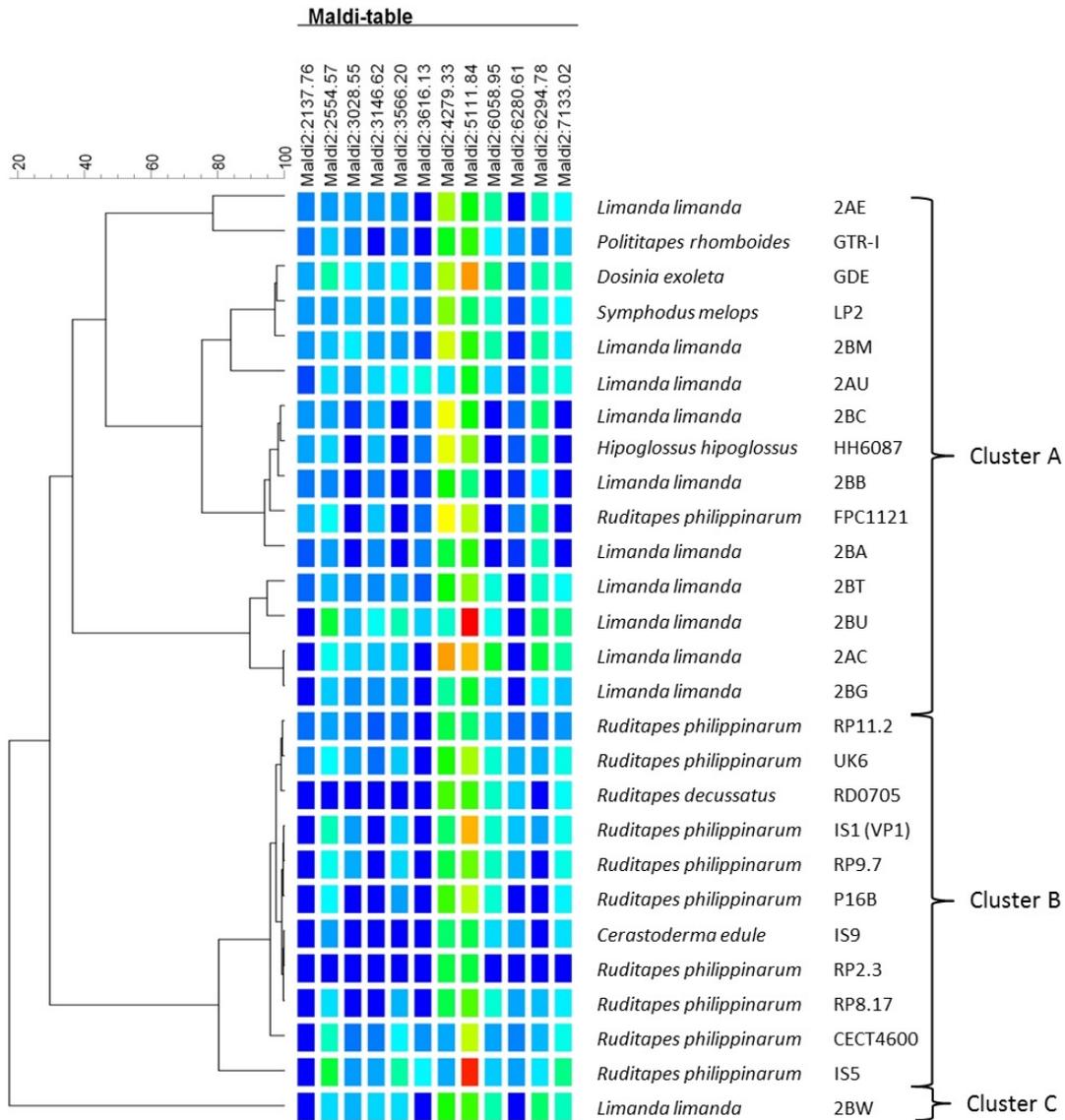
475

476 **Fig 1** Effect of isolates of *V. tapetis* from common dab on non-adherent cell ratio. Incubation time: 3h.

477 Results are presented by a mean ratio of non-adherent hemocytes (i.e. round hemocytes) in presence of

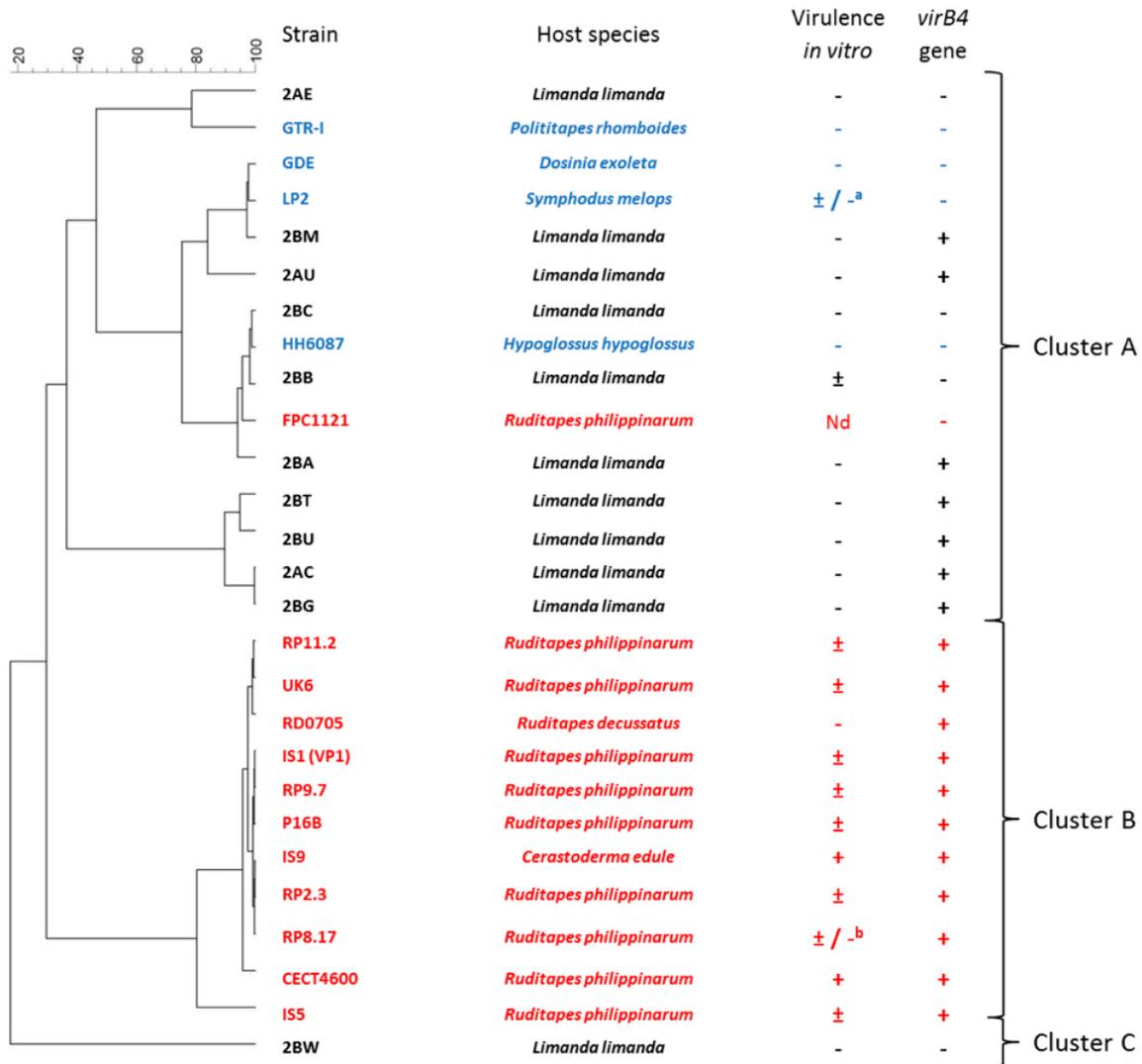
478 bacteria to the number of non-adherent cells after incubation with filtered sterile seawater (FSSW). Letters

479 depict significant differences (Pairwise Student test). Error bar = Standard Deviation/ \sqrt{n} (n = 3 replicates)



480

481 **Fig 2** Dendrogram, based on the MALDI-TOF peak lists, of all *V. tapetis* isolates based on the complete
 482 spectra. Peak intensity is represented in the heat map using different colors ranging from blue (low
 483 intensity), over green (low intermediate) to yellow (intermediate), orange (high intermediate) and red
 484 (high intensity).



485

486 **Fig 3** Dendrogram, based on the MALDI-TOF peak lists, of *V. tapetis* isolates derived from bivalves and fish

487 combined with *in vitro* virulence (hemocyte cytotoxicity assay) and presence of the *virB4* gene. In red:

488 isolates that can induce Brown Ring Disease *in vivo* after injection in the pallial cavity. In blue: isolates that

489 cannot induce Brown Ring Disease after injection in the pallial cavity. In black: isolates where no *in vivo*

490 assay has been performed against the Manila clam. References of the virulence profiles can be found in

491 Table 1. Cytotoxicity *in vitro* is depicted as follows: (-): no cytotoxicity, (±) intermediate cytotoxicity, (+):

492 comparable cytotoxicity as the reference strain (CECT4600). ^a: negative in Dias et al, 2018 but intermediate

493 in Choquet et al, 2003, ^b: negative in Dias et al, 2018 but intermediate in Choquet et al, 2004. Presence (+)

494 or absence (-) of the *virB4* gene as assessed using the TaqMan PCR method is also included. Nd: Not

495 determined