Aquaculture
January 2021, Volume 530, Pages 735729 (9p.)
https://doi.org/10.1016/j.aquaculture.2020.735729
https://archimer.ifremer.fr/doc/00641/75310/

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

Rahmani Alexandra [1, *], Vercauteren Maaike [1, 2], Vranckx Katleen [3], Boyen Filip, Bidault Adeline [4], Pichereau Vianney [4], Decostere Annemie [2], Paillard Christine [1], Chiers Koen [1, 2]

[1] Univ Brest, CNRS, IRD, Ifremer, UMR 6539 LEMAR, F-29280 Plouzane, France
[2] Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
[3] Applied Maths NV, Sint-Martens-Latem, Belgium
[4] Univ Brest, CNRS, IRD, Ifremer, UMR 6539 LEMAR, F-29280 Plouzane, France

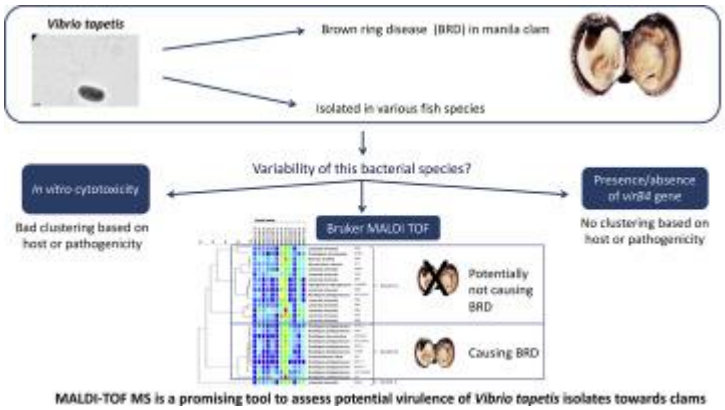
* Corresponding author : Alexandra Rahmani, email address : Alexandra.rahmani@univ-brest.fr

Maaike.vercauteren@ugent.be ; Katleen.vranckx@biomerieux.com ; filip.boyen@ugent.be ; adeline.bidault@univ-brest.fr ; Vianney.pichereau@univ-brest.fr ; annemie.decostere@ugent.be ; Christine.paillard@univ-brest.fr ; koen.chiers@ugent.be

Abstract :

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

Graphical abstract



MALDI-TOF MS is a promising tool to assess potential virulence of *Vibrio tapetis* isolates towards clams

Highlights

► Common dab's isolates of *V. tapetis* show very limited virulence *in vitro* ► This study has detected the *virB4* gene in non-cytotoxic fish isolates for the first time ► MALDI-TOF clustering seems to be a promising predictor for virulence of *V. tapetis'* strains ► New perspectives to study the virulence of *V. tapetis* isolates and to perform environmental monitoring to prevent outbreaks

Keywords : *Vibrio tapetis*, MALDI-TOF MS, virB4, Hemocyte cytotoxicity assay, Manila clam, Fish

ABBREVIATIONS

BRD: Brown ring Disease

FSSW: Filtered Sterilized Sea Water

HPLC: High performance liquid chromatography

67 MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

68 MLSA: Multilocus sequence analysis

69 RAPD-PCR: Randomly Amplified Polymorphic DNA PCR

70 T4SS: Type IV secretion system

71 TSA: Tryptic Soy Agar

72 TSB: Tryptic Soy Broth

73

74

1. INTRODUCTION

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

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

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

subsp. *britannicus* (Balboa and Romalde, 2013) and subsp. *quintayensis* (Levican et al., 2017). However, using genotyping methods, such as Randomly Amplified Polymorphic DNA (RAPD) PCR (Romalde et al., 2002) and multilocus sequence analysis (MLSA) (Gulla et al., 2017) different clusters were defined between *V. tapetis* subspecies. This indicates that the phylogeny of *V. tapetis* is not yet completely unraveled.

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

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

The main aim of the present study was to further explore the variability between the 27 *V. tapetis* isolates from bivalves and fish, considering three different aspects; *in vitro* virulence based on the loss of hemocyte adhesion properties after contact with *V. tapetis* (Choquet et al., 2003), detection of the *virB4* gene using the TaqMan qPCR (Bidault et al., 2015), and protein-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) characterization. Finally, these

approaches were compared and evaluated for their ability to discriminate the potential virulence of the 27 isolates against the Manila clams.

2. MATERIALS AND METHODS

2.1. Bacterial isolates and cultivation

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

For the cytotoxicity bioassay, only the isolates derived from common dab and the CECT4600 reference strain were used. All isolates were cultivated on Tryptic Soy Agar (TSA; Difco™) supplemented with 1.5 % NaCl and were allowed to grow for two days at 16 ± 1 °C (fish isolates) or 18 ± 1 °C (reference clam isolates). For the *virB4* gene detection only common dab isolates were used. All isolates were cultured in TSA supplemented with 1.6 % NaCl for PCR and Tryptic Soy Broth (TSB; Difco™) supplemented with 1.6 % NaCl for qPCR. For MALDI-TOF analysis, all 27 isolates were cultivated in triplicate (biological replicates, Supplementary file 1) on TSA supplemented with 1.5 % NaCl. Isolates were grown for minimally two days at 16 ± 1 °C (fish isolates) or 18 ± 1 °C (clam isolates). We chose to use the optimal or common temperatures for the strains to ensure optimal growth of the isolates.

2.2. In vitro hemocyte cytotoxicity bioassay

Virulence of *V. tapetis* isolates derived from common dab was tested using the standardized *in vitro* hemocyte cytotoxicity bioassay (Choquet et al., 2003). This test is based on the ability of *V. tapetis* virulent strains to induce a rounding phenotype on infected hemocytes, increasing the number of non-adherent as compared to the negative control. *V. tapetis* induces during its phagocytosis, a process of cytoskeletal inhibition and cell rounding. Thus, this adhesion test provides not only morphological but also functional characterization. The type strain CECT4600 was used as the positive control, and filter-sterilized seawater (FSSW) as negative control. Animals used in this study were Manila clams (4 cm

Manila clam, February 2018) from the SATMAR shellfish aquaculture site in Landeda (Finistère, France). Clams were allowed to acclimate in oxygenated seawater at 14°C for 14 days. Hemolymph was harvested from the adductor muscle, pooled after quality check and the hemocytes were enumerated using a Malassez counting grid. 100 µL of hemolymph (5. 10^5 hemocyte/mL) was added in 24-well plates and kept for a few minutes to let hemocytes adhere to the bottom of the plate. Then, 100 µl of bacterial suspension prepared with FSSW, was added at a bacteria/hemocyte ratio of 25/1. Hemocyte exposure to each of the bacterial isolates was performed in triplicate. In negative control samples, 100 µl of FSSW was added to the hemolymph. After 3 h of incubation, the number of non-adherent hemocytes was measured (used as a proxy of *V.tapetis* cytotoxicity) using flow cytometry after addition of 4 µl of SYBR–Green solution in DMSO (nucleic acid gel stain, dilution 1 : 10 000, Life Technology, USA) as already described (Choquet et al., 2003). A ratio was calculated by dividing the number of non-adherent hemocytes in samples exposed to bacteria by the number of non-adherent cells in negative controls. Statistical analyses were performed using a pairwise Student test to determine significant differences in non-adherent cell ratios between bacterial suspensions and both positive and negative control samples as already performed by Choquet et al. (2003).

2.3. VirB4 gene detection

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

2.4. MALDI-TOF MS characterization

An ethanol formic acid extraction was performed, based on MALDI Biotyper protocol (Bruker Daltonics, Bremen Germany). Briefly, bacterial cultures were suspended in nuclease-free water aliquoted in 1.5 mL

Eppendorf tubes. Ethanol (70 %) dissolved in high performance liquid chromatography (HPLC) grade water was added to the suspension and tubes were centrifuged twice (20 000 x g, 2 min) upon which the ethanol was removed. Thereafter, 20 µL of 70% formic acid (in HPLC grade water) was added to the pellet. To finish the extraction, 20 µL of acetonitrile was added. One µL of each extract was spotted eight-fold (technical replicates) on a MALDI target plate (Bruker Daltonics, Bremen, Germany), air dried and covered with 1 µL of alpha-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Bremen, Germany). All spots were processed in triplicate (technical replicates, Supplementary file 1) with an Autoflex III Smartbeam MALDI-TOF MS, recording masses ranging from 2 000 to 20 000 Da using standard settings (flexControl 1.4, version 3.4, Bruker Daltonic, Bremen, Germany). The obtained raw spectra were imported in BioNumerics 7.6.3 (Applied Maths NV, Sint-Martens-Latem, Belgium) for data analysis. Preprocessing of the data was performed according to (Giacometti et al., 2018). After preprocessing, peaks were detected using the continuous wavelet transform method with a signal-to-noise threshold of two. The spectra were summarized in an average spectrum per biological replicate (Supplementary file 1) and all replicates with less than 95% similarity to this summary spectrum were removed from the analysis. Of *V. tapetis* 2BB, RD0705 and RP2.3, only two biological replicates were implemented in the analyses due to inconsistencies in the data or low similarity (< 50%) with other replicates of the same isolate. The resulting summary spectra were used to construct an UPGMA dendrogram using a Pearson similarity coefficient.

3. RESULTS AND DISCUSSION

Infectious diseases remain one of the main limiting factors in the aquaculture of clams, therefore increasing the value of research on the variability of those infectious agents (Smits et al., 2020). The wide host (at least fourteen different host species) and geographic distribution (at least seven different countries) of *V. tapetis* and linked questions on possible variability of isolates have been discussed in previous research with a deep interest in finding a discriminative test for assessing pathogenicity of *V. tapetis* isolates against clams (Bidault et al., 2015). Furthermore, the discovery of new isolates from skin

199 ulcerations in the common dab (Vercauteren et al., 2018) might urge for further exploration of
200 pathogenic markers using available techniques.

201 ***3.1. Most of the V. tapetis isolates from the common dab are unable to induce hemocyte toxicity.***

202 To gain insight into the pathogenicity of *V. tapetis* to the Manila clam, an *in vitro* cytotoxicity assay was
203 developed based on the cell-rounding and subsequent loss of adherence of hemocytes following
204 exposure to *V. tapetis* (Choquet et al., 2003). This bioassay was previously performed for many *V. tapetis*
205 strains pathogenic to bivalves (Table 1), whereby strains IS9 and CECT4600 induced the highest loss of
206 adhesion of the hemocytes (i.e. *in vitro* cytotoxicity). Most of the other strains from clams displayed
207 intermediate cytotoxicity and only a few showed no cytotoxicity (Table 1). Remarkably, the GDE and
208 GTR-I strains, both isolated from bivalves but not from the Manila clam, showed no cytotoxicity *in vitro*.
209 Complete genome analyses of these two strains revealed strong differences with clam isolates (Dias et
210 al., 2018) and these two strains were genetically closer to the LP2 strain derived from corkwing wrasse.
211 This results further substantiates the observed difference in cytotoxicity towards Manila clam hemocytes
212 (Dias et al., 2018).

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

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

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

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

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

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

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

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

277 FPC1121 is the only clam pathogenic strain that was clustered together with fish isolates, based on the
278 MALDI-TOF MS profiling. FPC1121 was isolated from cultivated Manila clam in Japan (Table 1,
279 Matsuyama et al., 2010). The original report clearly described the brown deposit and mass mortalities of
280 Manila clam due to this strain, which was confirmed by *in vivo* virulence assays (Matsuyama et al., 2010).
281 This clustering of FPC1121 is an interesting result; detailed genetic analysis would be interesting to
282 explore the phylogenetic position of this strain and the linked genetic differences.

283 ***3.4. Value of the assays to differentiate isolates according to their host-species (Fig 3)***

284 *In vivo* virulence tests are necessary to determine the ability for an isolate to induce BRD. However,
285 several tests have been developed to characterize the *in vitro* virulence regarding Manila clam.
286 The *in vitro* cytotoxicity assay has been used as a tool to evaluate the pathogenicity of *V. tapetis* strains
287 for clams (Dias et al., 2018; Bidault et al., 2015). This assay is based on the correlation between the
288 cytotoxic activity of bacteria to clam hemocytes and the *in vivo* pathogenicity. As demonstrated in Table
289 1, such a correlation was not found for strain RD0705. Indeed, this strain causes no hemocytes
290 cytotoxicity *in vitro* although it can cause BRD *in vivo* in the Manila clam (Table 1) (Dias et al., 2018;
291 Novoa et al., 1998). Since RD0705 was isolated from another clam species (i.e. the grooved carpet shell),
292 it is tempting to speculate that this inconsistency might be related to host specificity. However, it could
293 also suggest that cytotoxicity towards hemocytes is not the only virulence factor involved in the
294 development of BRD. Beside this exception, it should be recalled that the analysis of the virulence
295 profiles of many *V. tapetis* strains isolated from clam revealed a good correlation between the cytotoxic
296 activity to clam hemocytes and the *in vivo* pathogenicity.
297 Another assay commonly used to detect *V. tapetis* isolates pathogenic to clams is the search for the *virB4*

298 gene encoding part of the T4SS (Dias et al., 2018). In our study, we have demonstrated for the first time
299 that the *virB4* gene can also be present in fish isolates. Since these isolates did not display *in vitro*
300 virulence to clam hemocytes and they clustered separately based on their protein profile, it could be
301 possible that they are not pathogenic for clams. *In vivo* studies should be performed to confirm the
302 pathogenicity towards clams and if so, this might question the use of *virB4* detection as a pertinent
303 marker for pathogenicity to clams.
304 The MALDI-TOF dendrogram showed a good clustering of *V. tapetis* from different origins. However, this
305 clustering was not correlated with the presence of the *virB4* gene. In fact, the clustering was more
306 correlated with the host species from which they were isolated. Based on the results of the different
307 assays in the present study, it could be hypothesized that MALDI-TOF clustering could differentiate
308 between clam pathogenic and nonpathogenic isolates.

309 ***3.5. Implications in the context of virulent strain detection***

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

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

319 **4. CONCLUSION**

320 In conclusion, the discovery of new *V. tapetis* isolates derived from common dab was linked with the
321 need for a further exploration of variability of *V. tapetis* isolates. The currently used assays (toxicity to

hemocytes and search of the *virB4* gene), have previously been shown to be interesting to differentiate between *V. tapetis* pathogenic and non-pathogenic clam isolates. Nevertheless, in this study, *V. tapetis* isolates from dab showed inconsistencies with results of previously pinpointed techniques questioning their discriminative power. In contrast, the MALDI-TOF MS analysis proved to be a promising tool with the possible ability to differentiate between pathogenic and non-pathogenic clam isolates. This can be used as a complementary discriminative test for virulence of clam isolates.

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

REFERENCES

Allam, B., Paillard, C., Auffret, M., 2000. Alterations in hemolymph and extrapallial fluid parameters in the Manila clam, *Ruditapes philippinarum*, challenged with the pathogen *Vibrio tapetis*. J. Invertebr. Pathol. 76, 63–69. https://doi.org/10.1006/jipa.2000.494

Allam, B., Paillard, C., Ford, S.E., 2002. Pathogenicity of *Vibrio tapetis*, the etiological agent of brown ring disease in clams. Dis. Aquat. Organ. 48, 221–231.

Allam, B., Paillard, C., Maes, P., 1996. Localization of the pathogen Vibrio P1 in clams affected by Brown Ring Disease. Dis. Aquat. Organ. 27, 149–155. https://doi.org/10.3354/dao027149

Balboa, S., Romalde, J.L., 2013. Multilocus sequence analysis of *Vibrio tapetis*, the causative agent of Brown Ring Disease: Description of *Vibrio tapetis subsp. britannicus subsp. nov*. Syst. Appl. Microbiol. 36, 183–187. https://doi.org/10.1016/j.syapm.2012.12.004

Bidault, A., Richard, G.G., Le Bris, C., Paillard, C., 2015. Development of a Taqman real-time PCR assay for rapid detection and quantification of *Vibrio tapetis* in extrapallial fluids of clams. PeerJ 3, e1484. https://doi.org/10.7717/peerj.1484

Borrego, J.J., Castro, D., Luque, A., Paillard, C., Maes, P., Garcia, M.T., Ventosa, A., 1996. *Vibrio tapetis sp. nov.*, the causative agent of the brown ring disease affecting cultured clams. Int. J. Syst. Evol. Microbiol. 46, 480–484.

Castro, D., Santamaria, J.A., Luque, A., Martinez-Manzanares, E., Borrego, J.J., 1996. Antigenic characterization of the etiological agent of the brown ring disease affecting manila clams. Syst. Appl. Microbiol. 19, 231–239. https://doi.org/10.1016/S0723-2020(96)80049-X

Choquet, G., 2004. Caractérisation et pathogénie des isolats de *Vibrio tapetis*, bactérie responsable de la maladie de l'anneau brun chez la palourde japonaise. Brest.

Choquet, G., Soudant, P., Lambert, C., Nicolas, J.-L., Paillard, C., 2003. Reduction of adhesion properties of *Ruditapes philippinarum* hemocytes exposed to *Vibrio tapetis*. Dis. Aquat. Organ. 57, 109–116.

Declercq, A.M., Chiers, K., Soetaert, M., Lasa, A., Romalde, J.L., Polet, H., Haesebrouck, F., Decostere, A., 2015. *Vibrio tapetis* isolated from vesicular skin lesions in Dover sole *Solea solea*. Dis. Aquat. Organ. 115, 81–86.

Dias, G.M., Bidault, A., Le Chevalier, P., Choquet, G., Der Sarkissian, C., Orlando, L., Medigue, C., Barbe, V., Mangenot, S., Thompson, C.C., Thompson, F.L., Jacq, A., Pichereau, V., Paillard, C., 2018. *Vibrio tapetis* Displays an Original Type IV Secretion System in Strains Pathogenic for Bivalve Molluscs. Front. Microbiol. 9, 227.

FAO (Ed.), 2018. Meeting the sustainable development goals, The state of world fisheries and aquaculture. Rome.

Figueras, A., Robledo, J.A.F., Novoa, B., 1996. Brown ring disease and parasites in clams (*Ruditapes decussatus* and *R. philippinarum*) from Spain and Portugal. J. Shellfish Res. 15, 363–368.

Giacometti, F., Piva, S., Vranckx, K., De Bruyne, K., Drigo, I., Lucchi, A., Manfreda, G., Serraino, A., 2018.

Application of MALDI-TOF MS for the subtyping of *Arcobacter butzleri* strains and comparison with their MLST and PFGE types. Int. J. Food Microbiol. 277, 50–57. https://doi.org/10.1016/j.ijfoodmicro.2018.04.026

Gulla, S., Rønneseth, A., Sørum, H., Vågnes, Ø., Balboa, S., Romalde, J.L., Colquhoun, D.J., 2017. *Vibrio tapetis* from wrasse used for ectoparasite bio-control in salmon farming: phylogenetic analysis and serotyping. Dis. Aquat. Organ. 125, 189–197. https://doi.org/10.3354/dao03140

Jensen, S., Samuelsen, O., Andersen, K., Torkildsen, L., Lambert, C., Choquet, G., Paillard, C., Bergh, Ø., 2003. Characterization of strains of Vibrio splendidus and V. tapetis isolated from corkwing wrasse Symphodus melops suffering vibriosis. Dis. Aquat. Organ. 53, 25–31. https://doi.org/10.3354/dao053025

Levican, A., Lasa, A., Irgang, R., Romalde, J.L., Poblete-Morales, M., Avendaño-Herrera, R., 2017. Isolation of *Vibrio tapetis* from two native fish species (*Genypterus chilensis* and *Paralichthys adspersus*) reared in Chile and description of *Vibrio tapetis subsp. quintayensis subsp. nov*. Int. J. Syst. Evol. Microbiol. 67, 716–723. https://doi.org/10.1099/ijsem.0.001705

Maes, P., Paillard, C., 1992. Effect du *Vibrio* P1, pathogene de *Ruditapes philippinarum*, sur d'autres espèces de bivalves, in: Les Mollusques Marins: Biologie et Aquaculture, Brest (France), 9 Nov 1990.

Maier, T., Klepel, S., Renner, U., Kostrzewa, M., 2006. Fast and reliable MALDI-TOF MS–based microorganism identification. Nat. Methods 3, i–ii. https://doi.org/10.1038/nmeth870

Matsuyama, T., Sakai, T., Kiryu, I., Yuasa, K., Yasunobu, H., Kawamura, Y., Sano, M., 2010. First Isolation of *Vibrio tapetis*, the Etiological Agent of Brown Ring Disease (BRD), in Manila Clam *Ruditapes philippinarum* in Japan. Fish Pathol. 45, 77–79. https://doi.org/10.3147/jsfp.45.77

Novoa, B., Luque, A., Castro, D., Borrego, J.J., Figueras, A., 1998. Characterization and Infectivity of Four Bacterial Strains Isolated from Brown Ring Disease-Affected Clams. J. Invertebr. Pathol. 71, 34–41. https://doi.org/10.1006/jipa.1997.4704

Paillard, C., 2016. An ecological approach to understanding host-pathogen-environment interactions: the case of Brown Ring Disease in clams, in: Oysters and Clams: Cultivation, Habitat Threats and Ecological Impact. ed. Jesus L. Romalde Microbiology research advances, Nova Science Publishers, p. 228.

Paillard, C., 2004a. Rôle de l'environnement dans les interactions hôtes-pathogènes; développement d'un modèle de vibriose chez les bivalves. Habilit. À Dir. Rech. HDR Univ. Bretagne Occident. Brest.

Paillard, C., 2004b. A short-review of brown ring disease, a vibriosis affecting clams, *Ruditapes philippinarum* and *Ruditapes decussatus*. Aquat. Living Resour. 17, 467–475. https://doi.org/10.1051/alr:2004053

Paillard, C., Maes, P., 1995. The brown ring disease in the Manila clam, *Ruditapes philippinarum*: I. Ultrastructural alterations of the periostracal lamina. J. Invertebr. Pathol. 65, 91–100.

Paillard, C., Maes, P., 1990. Aetiology of brown ring disease in *Tapes philippinarum*: pathogenicity of a *Vibrio sp.* Comptes Rendus Académie Sci. Ser. 3 Sci. Vie 310, 15–20.

Paillard, C., Maes, P., Oubella, R., 1994. Brown ring disease in clams. Annu. Rev. Fish Dis. 4, 219–240. https://doi.org/10.1016/0959-8030(94)90030-2

Reid, H.I., Duncan, H.L., Laidler, L.A., Hunter, D., Birkbeck, T.H., 2003. Isolation of *Vibrio tapetis* from cultivated Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture 221, 65–74.

Rodríguez, J.M., López-Romalde, S., Beaz, R., Alonso, M.C., Castro, D., Romalde, J.L., 2006. Molecular fingerprinting of *Vibrio tapetis* strains using three PCR-based methods: ERIC-PCR, REP-PCR and RAPD. Dis. Aquat. Organ. 69, 175–183.

Romalde, J.L., Castro, D., Magariños, B., Lopez-Cortes, L., Borrego, J.J., 2002. Comparison of ribotyping, randomly amplified polymorphic DNA, and pulsed-field gel electrophoresis for molecular typing of *Vibrio tapetis*. Syst. Appl. Microbiol. 25, 544–550.

Sandrin, T.R., Goldstein, J.E., Schumaker, S., 2013. MALDI TOF MS profiling of bacteria at the strain level: A review. Mass Spectrom. Rev. 32, 188–217. https://doi.org/10.1002/mas.21359

Singhal, N., Kumar, M., Kanaujia, P.K., Virdi, J.S., 2015. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.00791

Smits, M., Artigaud, S., Bernay, B., Pichereau, V., Bargelloni, L., Paillard, C., 2020. A proteomic study of resistance to Brown Ring disease in the Manila clam, Ruditapes philippinarum. Fish Shellfish Immunol. 99, 641–653. https://doi.org/10.1016/j.fsi.2020.02.002

Vercauteren, M., De Swaef, E., Declercq, A.M., Polet, H., Aerts, J., Ampe, B., Romalde, J.L., Haesebrouck, F., Devriese, L., Decostere, A., Chiers, K., 2019. Scrutinizing the triad of *Vibrio tapetis*, the skin barrier and pigmentation as determining factors in the development of skin ulcerations in wild common dab (*Limanda limanda*). Vet. Res. 50, 41. https://doi.org/10.1186/s13567-019-0659-6

Vercauteren, M., Swaef, E.D., Declercq, A., Bosseler, L., Gulla, S., Balboa, S., Romalde, J.L., Devriese, L., Polet, H., Boyen, F., Chiers, K., Decostere, A., 2018. First isolation of *Vibrio tapetis* and an atypical strain of *Aeromonas salmonicida* from skin ulcerations in common dab (*Limanda limanda*) in the North Sea. J. Fish Dis. 41, 329–335. https://doi.org/10.1111/jfd.12729

ACKNOWLEDGEMENTS

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

AUTHORS CONTRIBUTIONS

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

CONFLICTS OF INTEREST

Alexandra Rahmani declares that she has no conflict of interest.

Maaike Vercauteren declares that she has no conflict of interest.

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

Filip Boyen declares that he has no conflict of interest.

Adeline Bidault declares that she has no conflict of interest.

Vianney Pichereau declares that he has no conflict of interest.

Annemie Decostere declares that she has no conflict of interest.

Christine Paillard declares that she has no conflict of interest.

Koen Chiers declares that he has no conflict of interest.

FUNDING

The research was funded by the European Fisheries Fund (EVF – project VIS/15/A03/DIV), the Flemish Government and the Research Foundation – Flanders (FWO). This work makes use of the resources,

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

Compliance with Ethical Standards

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

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

462 **TABLES**

463 **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
464 health 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
465 (1) *in vivo* virulence, estimating the possibility of the isolate to cause Brown Ring Disease in Manila clam following experimental infection. The isolate
466 indicated with 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
467 clam ((-): no cytotoxicity, (±) intermediate cytotoxicity, (+):cytotoxicity. a: negative in Dias et al, 2018 and intermediate in Choquet, 2004. b: negative in Dias
468 et al, 2018 and intermediate in Choquet et al, 2003 And (3) presence of the *virB4* gene from Bidault et al., 2015; (+) : presence, (-): absence. References for
469 the virulence 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; *:
470 Determined in this study.

471

472

473

474

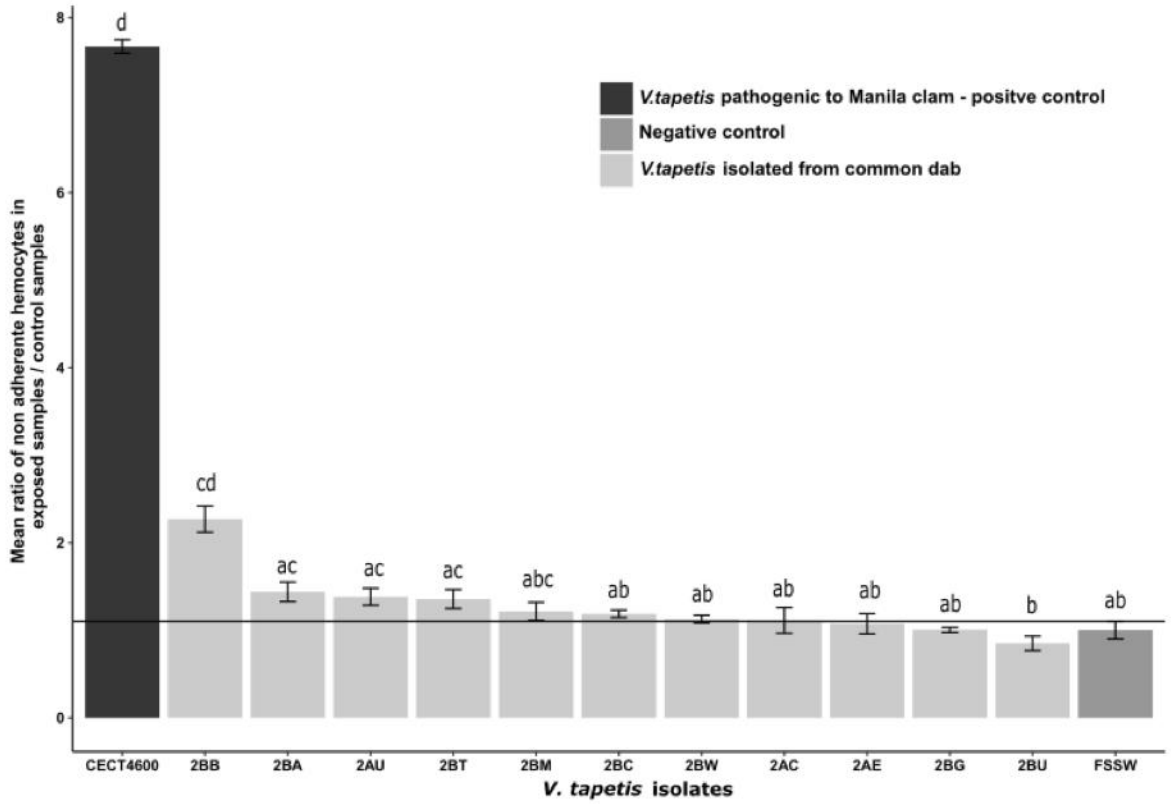
475

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

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

476

477 **FIGURES**



478

479 **Fig 1** Effect of isolates of *V. tapetis* from common dab on non-adherent cell ratio. Incubation time: 3h.
480 Results are presented by a mean ratio of non-adherent hemocytes (i.e. round hemocytes) in presence of
481 bacteria to the number of non-adherent cells after incubation with filtered sterile seawater (FSSW).
482 Letters depict significant differences (Pairwise Student test). Error bar = Standard Deviation/ √n (n = 3
483 replicates)

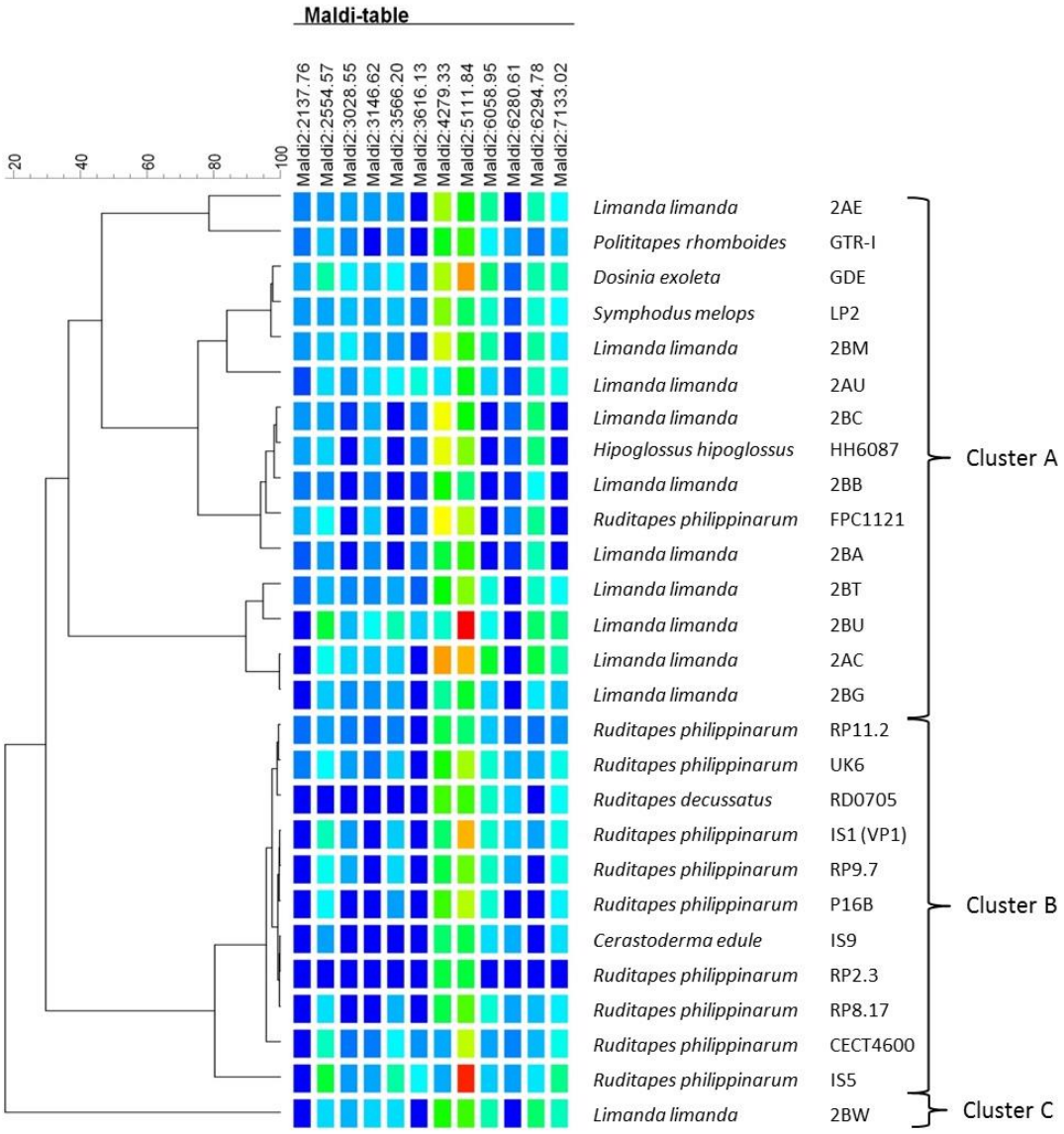


Fig 2 Dendrogram, based on the MALDI-TOF peak lists, of all *V. tapetis* isolates based on the complete spectra. Peak intensity is represented in the heat map using different colors ranging from blue (low intensity), over green (low intermediate) to yellow (intermediate), orange (high intermediate) and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

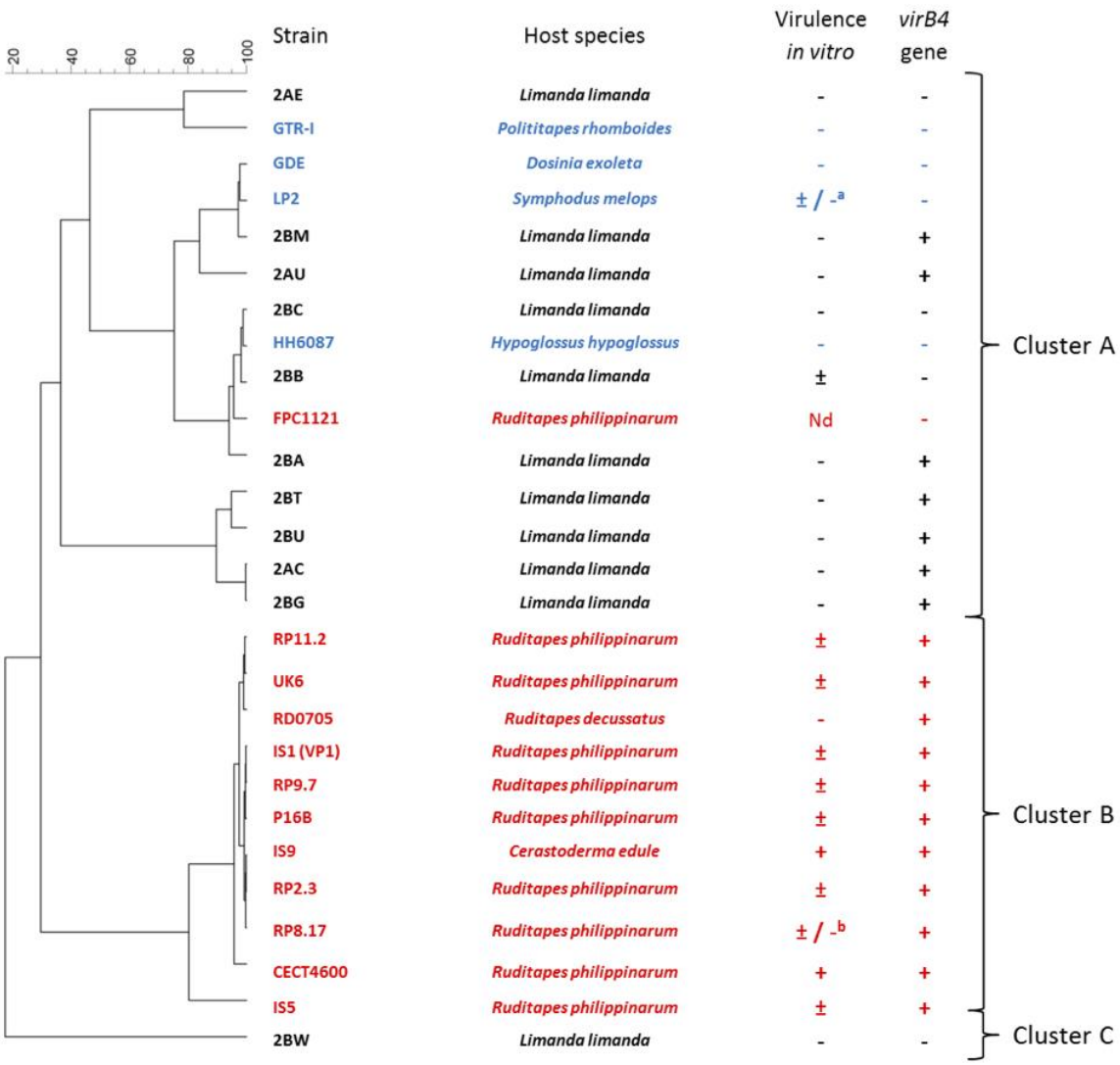


Fig 3 Dendrogram, based on the MALDI-TOF peak lists, of *V. tapetis* isolates derived from bivalves and fish combined with *in vitro* virulence (hemocyte cytotoxicity assay) and presence of the *virB4* gene. In red: isolates that can induce Brown Ring Disease *in vivo* after injection in the pallial cavity. In blue: isolates that cannot induce Brown Ring Disease after injection in the pallial cavity. In black: isolates where no *in vivo* assay has been performed against the Manila clam. References of the virulence profiles can be found in Table 1. Cytotoxicity *in vitro* is depicted as follows: (-): no cytotoxicity, (±) intermediate cytotoxicity, (+): comparable cytotoxicity as the reference strain (CECT4600). [a]: negative in Dias et al, 2018 but intermediate in Choquet et al, 2003, [b]: negative in Dias et al, 2018 but intermediate in Choquet et al, 2004. Presence (+) or absence (-) of the *virB4* gene as assessed using the TaqMan PCR method is also included. Nd: Not determined

500

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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

All other co-authors declare that they have no conflict of interest.

514 ***AUTHORS CONTRIBUTIONS***

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

518

519

520 **HIGHLIGHTS**

521 Common dab's isolates of *V. tapetis* show very limited virulence *in vitro*

522 This study has detected the *virB4* gene in non-cytotoxic fish isolates for the first time

523 MALDI-TOF clustering seems to be a promising predictor for virulence of *V. tapetis'* strains

524 New perspectives to study the virulence of *V. tapetis* isolates and to perform environmental monitoring
525 in order to prevent outbreaks