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Protocols

Genetic identification of two Acipenser iridovirus-European variants using high-resolution melting analysis

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ABSTRACT

Acipenser iridovirus-European (AcIV-E) is an important pathogen of sturgeons. Two variants differing by single-nucleotide polymorphisms (SNP) in the Major Capsid Protein gene have been described, but without any indication as to their prevalence in farms. To facilitate epidemiological studies, we developed a high-resolution melting (HRM) assay to distinguish between two alleles (var1 and var2) differing by five point substitutions. The HRM assay detected as little as 100 copies of plasmids harboring cloned sequences of var1 and var2, which have melting temperatures (Tm) differing by only 1 °C. The assay was specific of AcIV-E as demonstrated by the absence of signal when testing a related, yet distinct, virus as well as DNA from an AcIV-E-negative sturgeon sample. Experiments with mixtures of two distinct plasmids revealed abnormal melting curve patterns, which showed dips just before the main melting peaks. These dips in the curves were interpreted as the dissociation of heteroduplexes fortuitously created during the PCR step. Screening AciV-E-positive field samples of Russian sturgeons from three farms revealed the presence of var2, based on the Tm. However, for a few samples, the melting curves showed patterns typical of var2 as the dominant viral genome, mixed with another minor variant which proved to be var1. In conclusion, HRM is a simple method to screen for AcIV-E var1 and var2 and can be used on a large scale in Europe to trace these two variants which likely represent two genetic lineages.

1. Introduction

Acipenser iridovirus-European (AcIV-E) is a nucleocytoplasmic large DNA virus (NCLDV) infecting various sturgeon species in Europe (Bigarré et al., 2017; Ciulli et al., 2016). Its complete genome sequence has not been established; to date, only a 6 kb sequence encompassing the major capsid protein (MCP) gene and a replication factor C (RFC) gene is available (Pallandre et al., 2018). Based on this sequence, this virus has been shown to be related to other NCLDV sturgeon viruses found on the American continent, for instance the white sturgeon iridovirus (WSIV) and Namao virus (NV) (Bigarré et al., 2017; Clouthier et al., 2018; Pallandre et al., 2018). Since their initial observation by electron microscopy, these sturgeon NCLDVs have been classified in the *Iridoviridae* family, based on the shape of their particles (Clouthier et al., 2013; Hedrick et al., 1990). However, their MCP gene is very different from all other fish iridoviruses, and shows more genetic relationships with sequences from the *Mimiviridae* family, which are also members of the order *Megavirales*, as are NCLDVs (Colson et al., 2013). Recent genomic data confirmed the striking relationships of these fish viruses with *Mimiviridae* (Clouthier et al., 2018; Pallandre et al., 2018). Thus, it is likely that these sturgeon viruses will be reclassified and renamed in the near future.

To date, preliminary data indicate that AcIV-E is prevalent in European farmed sturgeons where it is associated with variable levels of mortality and morbidity, Russian sturgeon (*Acipenser gueldenstaedtii*) being particularly susceptible. The impact of the virus varies greatly, depending on the host species, the age of the fish, the presence of other pathogens and other factors still to be identified. The genotype of the virus probably also influences virulence although poor data is available about the genetic diversity of AcIV-E.

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In a previous study, two variants of AcIV-E showed single-nucleotide polymorphisms (SNPs), differing by at least five nucleotides in a particular region of the MCP gene (Bigarré et al., 2017). One variant (var1) carries the sequence $A_{GCCAATGT}$AAAC and the second variant (var2) has the sequence $C_{GCCAATGT}$GCTT. In a very small initial sample, the var2 allele was commonly found, whereas var1 was found in only three samples in co-infection with var2 in two host species from two countries. It is not known whether these two alleles reflect the existence of two viral lineages with many additional differences in the rest of the genomes or are simply variants of a unique lineage. However, the analysis of the RFC gene within a same viral population also strongly suggested the circulation of two lineages (Pallandre et al., 2018). In this view, the epidemiological analysis of molecular marker in the MCP is of interest to screen for a putative correlation between the lineages and virulence properties, or the lineages and geographic origin.

High-resolution melting (HRM) is a method commonly used for distinguishing SNPs in pathogens (Tong and Giffard, 2012). After amplification of a selected genomic region, a slow heat-driven fusion is carried out during which the fluorescence emitted by an intercalating (preferentially saturating) dye decreases as the DNA progressively switches from the double-stranded to the single-stranded state. The temperature at which half of the double-stranded DNA has melted (melting temperature, Tm) is specific to a particular primary sequence. HRM has been used for distinguishing species within a genus or variants within a species for both RNA and DNA viruses (Gelaye et al., 2017; Lieveld et al., 2017; Tong and Giffard, 2012; Towler et al., 2010). This type of analysis requires a less laborious method than the sequencing of PCR products.

To distinguish between the two known variants of AcIV-E in infected tissues, we developed an HRM-based assay.

2. Materials and methods

2.1. Samples

Most samples were from Russian sturgeons collected between 2016 and 2017. Gills, fins and/or mucus were collected from dead or moribund fish captured in three different French farms (F1, F2, F3) (Table 1). Two samples from 2015 (15/52 and 15/56) were used only to produce plasmids containing a portion of the viral genome.

2.2. Nucleic acid extraction from tissues and diagnostics

For one fish sampled in 2015 (15/52), total DNA was extracted at ANSES, using a NucleosSpin Blood kit (Macherey-Nagel) (Bigarré et al., 2017). Total DNAs from fish samples from 2016 were extracted at the Laboratoires des Pyrénées et des Landes using a NucleoSpin Tissue kit (Macherey-Nagel). Briefly, about 0.1 g of pooled organs (gills and fins) was crushed in lysis buffer using ceramic beads and a tissue homogenizer (Precellys 24, Bertin). A volume of 25 µL of this homogenate was incubated for 3 h at 56 °C with proteinase K. Nucleic acids (NA) were eluted in 100 µL of elution buffer. For samples from 2017, extracted at ANSES, organs were ground in PBS buffer (1:10, w/v) in Lysing Matrix D tubes (MP Biomedicals) and shaken for 20 s in a FastPrep FP120 homogenizer (ThermoFisher). After a centrifugation at 5000 g for 3 min, 200 µL of the supernatants were extracted using the Nucleospin Virus kit (Macherey-Nagel) according to the provided protocol, except that the incubation with proteinase K was carried out for 15 min at 70 °C instead of 3 min at room temperature. Elution was performed in 50 µL of RNase-free water. Extracted nucleic acids (DNA and RNA) were quantified by spectrophotometry with a Nanodrop 1000 (ThermoFisher). The presence of viral DNA was tested by real-time PCR with a Taqman probe as previously described (Bigarré et al., 2017).

Table 1
Origin, number and virus-positive (Taqman PCR) samples of this study.

Year	Farm	Number of assessed samples	Number of positive samples	Number of co-infected samples
2016	Farm 1	17	17	1
	Farm 2	15	15	0
2017	Farm 1	78	30	3
	Farm 2	83	53	3
	Farm 3	157	13	1
	Total	350	128	8

2.3. Plasmid construction and sequencing

To evaluate the sensitivity of the PCR and the Tm of the product of each variant, two plasmids previously constructed in 2015 were used (Bigarré et al., 2017). pPVP130 is a PCR4-TOPO plasmid (Invitrogen) in which was inserted a 1344 bp-PCR product containing a large portion of the AciV-E MCP gene (sample 15/52, allele var1) amplified with primers oPVP339 and oPVP344 (Bigarré et al., 2017). pPVP131 is a similar plasmid with a PCR product from another sample (15/56, allele var2). Both plasmids were retransformed in *Escherichia coli* in 2017 to produce fresh solutions using a Nucleospin Plasmid kit (Macherey-Nagel). Plasmids were quantified with a spectrophotometer SmartSpec™ 3000 (BioRad). Sanger sequencing revealed a total of 15 point variations between the viral sequence inserts, of which five were within the region amplified by PCR HRM, while the 10 others were outside this region. Therefore, the two amplicons differed only by five substitutions.

For the analysis of the intra-sample viral diversity by clone sequencing, conventional PCR (cPCR) was performed on samples with the forward oPVP339 and reverse oPVP340 primers (Bigarré et al., 2017). This pair of primers targets a 636 bp portion of the MCP gene, including the variable region of interest. In a total volume of 50 µL, 500 ng of total DNA were used with 0.4 mM of each primer, 1.5 µM of $MgCl_2$, 200 µM of dNTP and 2.5 U of TaqGold polymerase (Applied Biosystems). The following cycles were applied: 1 step of 8 min at 94 °C followed by 40 cycles at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min A volume of 10 µL was run on a precast 2% agarose E-gel (Invitrogen) for 15 or 30 min before observation under UV light. The 40 µL left were cleaned and eluted in 30 µL using a Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel). A volume of 2 µL of the eluted product was used for TA cloning (Invitrogen). Plasmids from individual clones were purified using the Nucleospin Plasmid kit (Macherey-Nagel), and then sequenced using the Sanger method and a 3130 Genetic Analyzer (Applied Biosystems). Sequences were verified and edited with VectorNTI11 (ThermoFisher).

2.4. HRM analysis

The forward oPVP440 (5' GTCTGAGGCCTTTGATGCAG 3') and reverse oPVP441 (5' AGCGCTTTCAACATTCAACTT 3') primers were designed to generate a 102 bp PCR product from the MCP gene, encompassing the variable region containing the SNPs. A PCR reaction was performed in a total volume of 20 µL with 5 µL of a plasmid solution or 1 to 2 µL of total nucleic acids from fish at 100 ng/µL and 400 nM of each HRM primer were used with the EvaGreen mix (Bio-Rad) as recommended. On a Bio-Rad CFX96 thermocycler or on an Applied Biosystems QuantStudio5 (QS5), the amplifications were run using the following protocol: 98 °C for 2 min followed by 40 cycles of 98 °C for 5 s and 60 °C for 5 s. Then, the double-stranded products were dissociated by ramping up the temperature from 65 °C to 95 °C with a 0.1 °C step every 5 s. All assays were performed in duplicates. For each run, a negative control (water) and two distinct positive controls (plasmids pPVP130 and pPVP131) were added, each in duplicates.

To test the specificity of the HRM primers, sturgeon samples producing no signal by taqman PCR were assessed. Additionally, a European sample (14/117) positive for a variant of NV was tested (Bigarré et al., 2017). Similarities with the published partial sequences

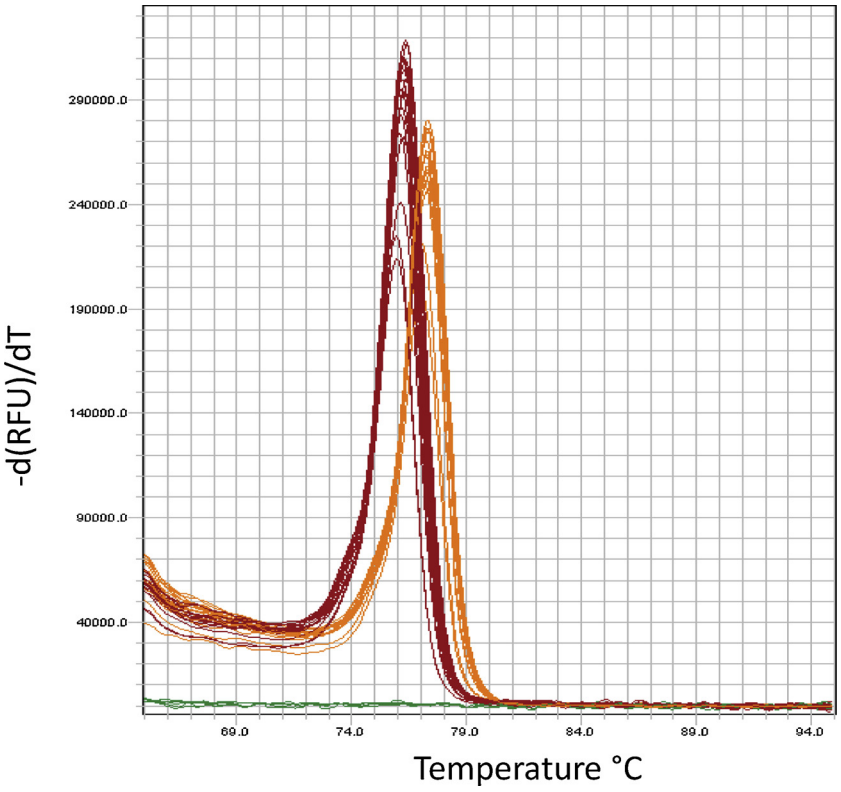


Fig. 1. Derivatives of the HRM assays using two plasmids carrying viral sequences of var1 and var2. Dilutions (10 to 10^8) of plasmid were assayed. Orange curves: var2; red curves: var1; green: negative control (water). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

of other NCLDV were searched by aligning DNA sequences: WSIV (Genbank DQ897645), NV from Canada (JX155659), NV from Europe (KU301309) and Acipenser herpesvirus type 2 from USA (FJ815289).

In a first step to evaluate the analytical sensitivity, serial 10-fold dilutions (10^8 to 1) of each pure solutions of plasmids were assayed in duplicates using PCR-HRM in one session. In a second step, three selected concentrations (10^2, 10 and 1) of both pure solutions of plasmids replications were tested over series of sessions. The solutions of 100 and 10 copies were tested in 42 repeats over eight sessions with the QS5 while the one-copy solution was tested in 18 replicates over five sessions. These same three concentrations were also used with 200 ng of total nucleic acids of a virus-negative sample in order to test the effect of host nucleic acids on the analytical sensitivity. A total of 24 replicates per solution was tested over 3 sessions.

In order to mimic co-infections of var1 and var2, various ratios (80:1; 40:1; 20:1; 10:1; 5:1; 1:1; 1:5; 1:10; 1:20; 1:40; 1:80) of pure plasmids (diluted at 10^4 copies/µl) were mixed, with (200 ng) or without total host nucleic acids from a virus-negative sturgeon.

Amplification and melting curve data were generated and analyzed using CFX manager 3.1 (Bio-Rad) and QuantStudio Design & Analysis software v1.4.2 (Applied Biosystems). In addition, the High-Resolution Melt software v3.1 (Applied Biosystems) was used to obtain the normalized curves.

3. Results

3.1. HRM specificity

A series of Russian sturgeon samples (48), previously determined as negative for AcIV-E with real-time TaqMan PCR, produced no signal in the PCR coupled with the HRM (PCR-HRM) assay (not shown). This result demonstrates that there is no cross-reaction of the HRM primers with the genome of the Russian sturgeon.

The HRM primers did not match the partial genome (66 kb) available for Acipenser herpesvirus 2, which is a pathogen infecting several sturgeon species (Doszpoly et al., 2017, 2011). To estimate the possibility of cross-reactions of the HRM primers with homologous regions of viruses related to AcIV-E, the sequences of the oligonucleotides were aligned with the MCP sequences of NV and WSIV. The HRM primers had a total of 17 mismatches (7 + 10) with WSIV and therefore would not amplify this portion of the genome under the stringent PCR conditions we used. Aligning with two MCP sequences of NV (from Canada and Europe), one HRM primer harbored only one mismatch, and the second had seven mismatches. The possibility of amplification was therefore very low, according to general recommendations for quantitative PCR (Lefever et al., 2013). Nevertheless, to verify non-amplification, PCR-HRM was performed on a fish sample infected with a European variant of NV. No signal was produced demonstrating the absence of cross-reactions between the HRM primers and the tested variant of NV (not shown).

3.2. Analytical sensitivity of HRM

Serial 10-fold dilutions (10^8 to 1) of plasmids pPVP130 and pPVP131 were assayed using PCR-HRM. When one complete series was tested for each plasmid, the lower limits of detection were 10 copies for plasmids pPVP 130 and pPVP131 (R^2 = 0.999 and 0.996; efficiencies = 108.4% and 105.5%, respectively for the QS5 thermocycler; R2 = 0.997 and 0.998; efficiencies = 98.7% and 100.7% respectively for the CFX96). With more replicates for three selected concentrations, pPVP130 produced a positive signal for all repeats (42) at 100 copies and for 41 out of 42 repeats at 10 copies (97.6%). With the plasmid pPVP131, a positive signal was produced at a frequency of 97.6% (41/42) at 100 copies and 78.5% (33/42) at 10 copies. When adding 1 to 100 copies of each plasmid to total nucleic acids from a virus-negative fish sample, similar sensitivities were obtained compared to pure plasmids: 100 copies were always detected for both plasmids (24/24), 10 copies detected at a frequency of 100% and 71% (24/24 and 17/24 for pPVP130 and pPVP131 respectively) and 1 copy poorly detected (8 and 4%). Therefore, the analytical sensitivity was at least 100 copies of plasmids. In addition, a series of 10-fold dilutions (100 ng to 1 pg) of nucleic acids from one virus-positive sample was tested by HRM,

producing efficiencies of 106.2% (R^2 = 0.997) and 108.9% (R^2 = 0.994) with the QS5 and CFX96 respectively. Therefore, the plasmids gives comparable results to those obtained with the viral genome mixed with the host NA.

For plasmid copy numbers between 10^8 and 10, the mean Tm values obtained for the var1 and var2 were 75.3 (± 0.19) and 76.3 (± 0.35) respectively, using CFX96, and were 76.2 (± 0.13) and 77.2 (± 0.09) using QS5 (Fig. 1). Thus, for a same thermocycler, the Tm values of the two distinct amplicons differed by 1 °C. For a same amplicon, the Tm values differed by 0.9 °C depending on the used thermocycler. Therefore, the two alleles were distinguishable by HRM. The Tm values obtained for different quantities of a given plasmid were almost identical at 10^3 copies and above, but dropped slightly at lower copy numbers.

3.3. HRM with plasmids mixed in different ratios

To simulate possible co-infections of viral variants within a same fish sample, controlled mixtures of plasmids were assayed using the HRM protocol. Different ratios of the two plasmids were analyzed using PCR-HRM. At all ratios, only one peak was observed on the melting curves between 73 and 80 °C, instead of the two peaks expected considering the presence of two distinct plasmid amplicons (Fig. 2a and b). Most likely, the resolution of the method was not high enough to separate the two peaks differing by only by 1 °C. Noticeably, at a 1:1 ratio, the unique peak was wider than those of the pure plasmids used as controls and showed a Tm value of 76.8 °C (QS5 platform), intermediate to the two expected individual Tm values (Fig. 2b). At other (asymmetric) ratios (1:80 to 1:5), the observed Tm values were nearly identical to those of the most abundant plasmid (Fig. 2a). Therefore, the observation of a unique melting peak did not always indicate the presence of a single amplicon.

However, for ratios of 1:40 to 1:1, the melting curves showed an unusual pattern at temperatures lower than 73 °C. Between about 70 and 73 °C, thus at temperatures lower than the melting point of the dominant amplicon, a sudden dip occurred in the curves (Fig. 2a, 2c). The different shapes of the curves obtained with these ratios and those of the positive controls were particularly visible after normalization using var1 as a reference (Fig. 2d). We interpreted this dip as the dissociation of a population of double-stranded (ds) DNA, which was likely composed of unstable heteroduplexes fortuitously created during the PCR. If this interpretation is correct, this dip in the curve simply indicates the presence of a mixture of distinct sequences in the DNA before amplification. Furthermore, there is no visible dip in the curve at plasmid ratios of 1:80, which is probably below the detection limit of the heteroduplexes. To demonstrate a direct link between the dip in the curve and the presence of heteroduplexes, further experimental assays were performed. Equal quantities of each plasmid were amplified by PCR and we tested their products in different conditions. When the two individual products were mixed after the PCR phase and subsequently dissociated by HRM, there was no dip in the melting curve at values less than 71 °C (Fig. 3c), exactly as if the products had not been mixed at all (Fig. 3a). However, when the two individual products were mixed after the PCR, melted at 98 °C for 5 min, subsequently re-associated at 20 °C for 15 min and then finally assayed using HRM, the characteristic dip was observed (Fig. 3d), exactly as when the plasmids were mixed before the PCR (Fig. 3b). Therefore, the artificial creation of heteroduplexes by heat-denaturing dsDNA, mixing and re-associating single-stranded DNA of two distinct amplicons induced the appearance of a marked dip in the melting curve plot before the main fusion peak. It was therefore concluded that this dip was associated with of a mixture of distinct sequences present before amplification.

The same plasmids ratios (1:80 to 1:1) were also tested with the presence of host NA. The obtained Tm values were similar compared to those from mixes of pure plasmids, varying between 0.1 and -0.25 (data not shown). Compared to the positive controls (pure plasmids + NA), the Tm of the 1:1 ratios were intermediate as expected. For ratios 1:40 to 1:1, the curves showed the same dips than those observed with the plasmids without additional NA. Therefore, in our conditions, the presence of host NA did not prevent the detection of hybrids.

3.4. HRM with field samples

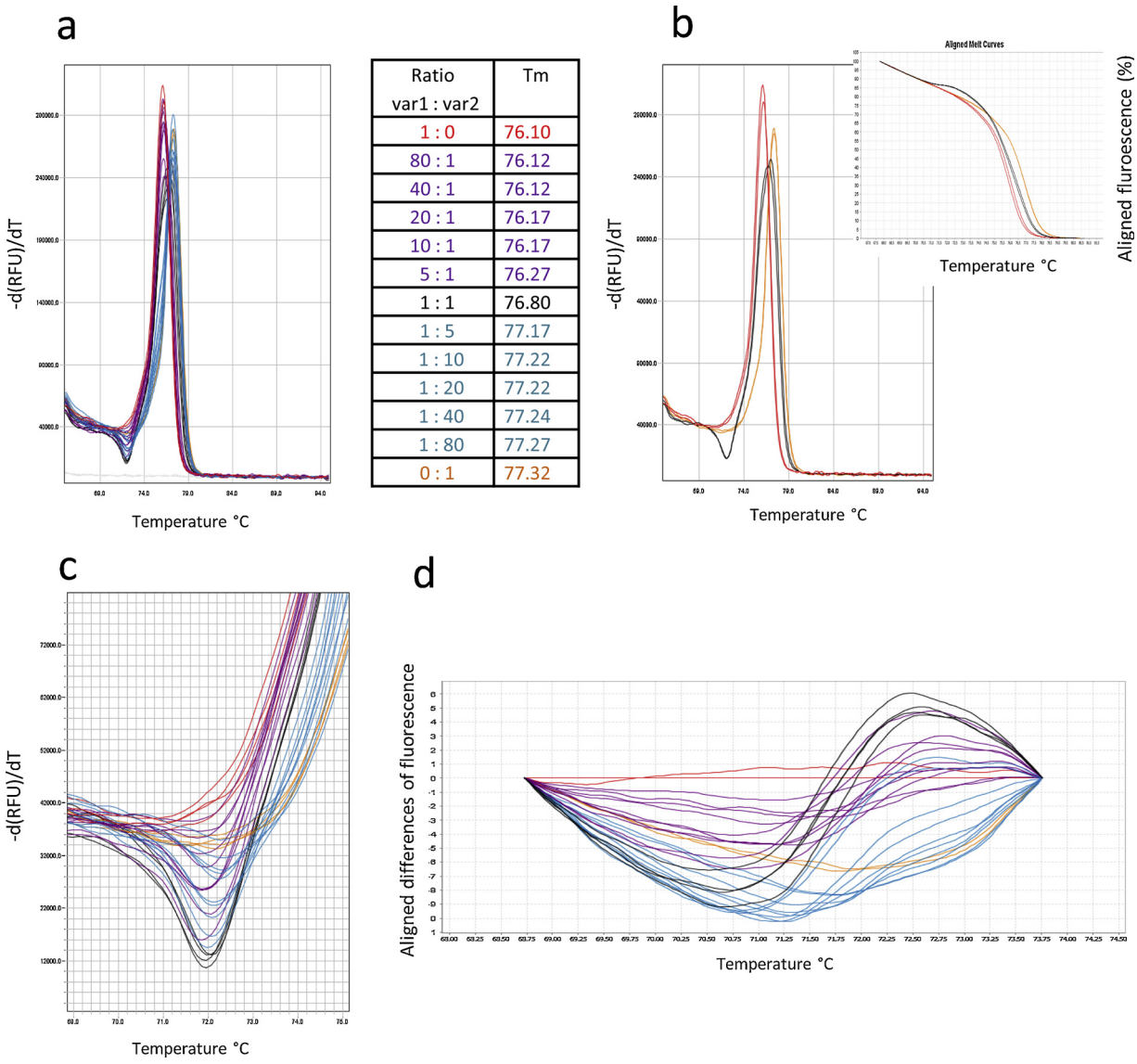
When testing field samples, the two plasmids were used at 10^4 copies in separate wells as positive controls in order to check the efficiency of the PCR and as a reference for the Tm of each variant. A series of field samples from 2016 and 2017, determined as positive for AcIV-E using TaqMan PCR, were tested using PCR-HRM. In the 128 samples, all produced Tm values indicating the presence of the var2 allele (Fig. 4). None of the samples produced the Tm expected for var1 alone. However, for seven samples from 2017, their melting curve exhibited a dip just before the fusion of the main product (Fig. 4a and b). The normalization of the curves before the main peak confirmed major differences of curve shapes between distinct samples, with two groups observed (Fig. 4c). One sample from 2016 also exhibited this dip (not shown). These observations strongly suggest the presence of another variant co-infecting these eight samples infected with var2. To verify this assumption, a cPCR (primers oPVP339 and oPVP340) was carried out on each of the eight fish samples, targeting a portion of the viral MCP gene. The products were cloned and several randomly chosen clones were sequenced. For each suspected co-infection, some clones corresponding to var1 and var2 were identified at various ratios, with var2 being the most frequently found (Table 2). In total, 21 clones harboring var1 and 94 clones with var2 were identified. Of the 21 clones corresponding to var1, 20 were strictly identical to the HRM region of pPVP130, whereas one exhibited a single mutation. Similarly, of the 94 clones of var2, 93 were identical to the HRM region of pPVP131, and one showed one substitution. However, regarding these two single changes, it was not possible to determine whether they were actual mutations persisting within the viral population or simply artefacts produced during the PCR step. Consistent with the findings in the eight samples from 2016 and 2017, a typical dip in the melting curve was also observed for sample 15/52 from 2015 for which the presence of var1 and var2 had previously been demonstrated (not shown).

These results showed that, for the studied period (2016–2017), var2 was the most frequently found allele in field samples and a few samples harbored a mixture of variants. Viral DNA with the var1 was found in the three studied farms.

4. Discussion

To date, the genetic diversity of sturgeon NCLDVs in Europe has been poorly studied. Two viruses have been found: AcIV-E found only in Europe and a second virus related to the Canadian NV. AcIV-E infects various sturgeon species in Europe and has been found in three countries. Whether this virus is endemic to Europe or has been imported *via* fish trade from another continent remains unknown. Regardless of its origin and considering its impact on fish production, it is important to study the evolution and dissemination of AcIV-E within Europe, ideally with the use of molecular markers. The AcIV-E MCP gene harbors a convenient molecular marker with two possible alleles, which may reflect the existence of two genetic lineages (Bigarré et al., 2017). If there are two lineages, this molecular marker will be useful to study their prevalence, providing that a simple genotyping method is available and routinely applicable on field samples. The proposed PCR-HRM assay can identify the presence of one or more MCP alleles at a reduced cost, without laborious sequencing.

Tests with plasmids harboring the var1 or var2 allele demonstrated the high sensitivity of the PCR-HRM, detecting as few as 10 copies, which is roughly equivalent to a specific TaqMan-based real-time PCR assay that we previously developed. For eight selected samples, the cycle threshold (Ct) values obtained by TaqMan PCR and PCR-HRM were in the same range. Therefore, the HRM can potentially be used as



Ratio var1 : var2	Tm
1 : 0	76.10
80 : 1	76.12
40 : 1	76.12
20 : 1	76.17
10 : 1	76.17
5 : 1	76.27
1 : 1	76.80
1 : 5	77.17
1 : 10	77.22
1 : 20	77.22
1 : 40	77.24
1 : 80	77.27
0 : 1	77.32

Fig. 2. Melting curves obtained with various ratios of two recombinant plasmids. a. derivatives curves obtained for 13 ratios of 2 plasmids. The table provides the correspondances between the colors and the curves, as well as the Tm values. b. same as a, with the pure plasmids and only one mix showed (1:1). The melting curves are shown in the frame. c. magnification of a) in the pre-melting region. d. Normalized difference fluorescences curves of the step (69–73 °C) before the main fusion peak; the var1 is used as a reference.

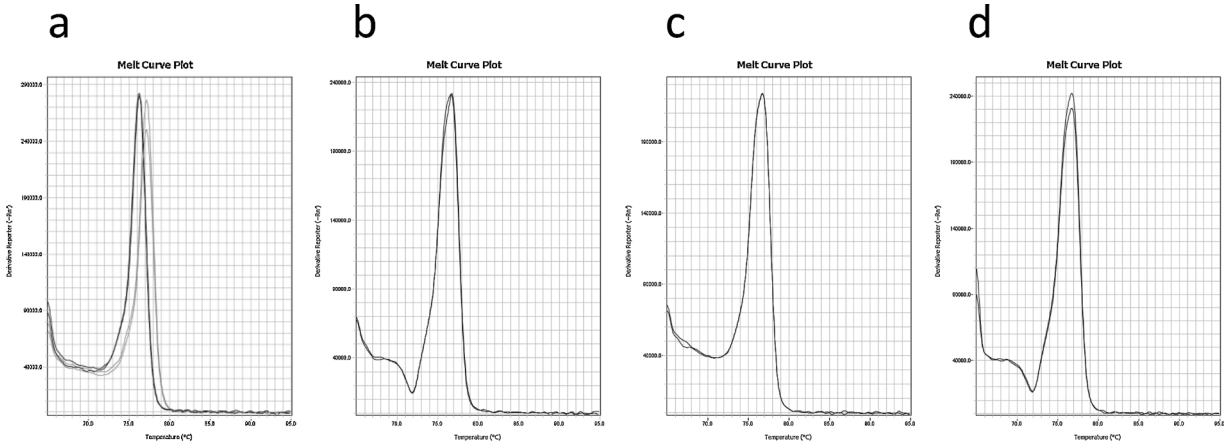


Fig. 3. Melting curves obtained with recombinant plasmids. a. var1 (black dot) and var2 (grey) amplified and melted in independant wells. b. var1 and var2 mixed before PCR and melting. c. var1 and var2 in independant wells for PCR; then amplicons were mixed before melting. d. var1 and var2 amplified in independant wells; then amplicons were mixed, heated, cooled and melted.

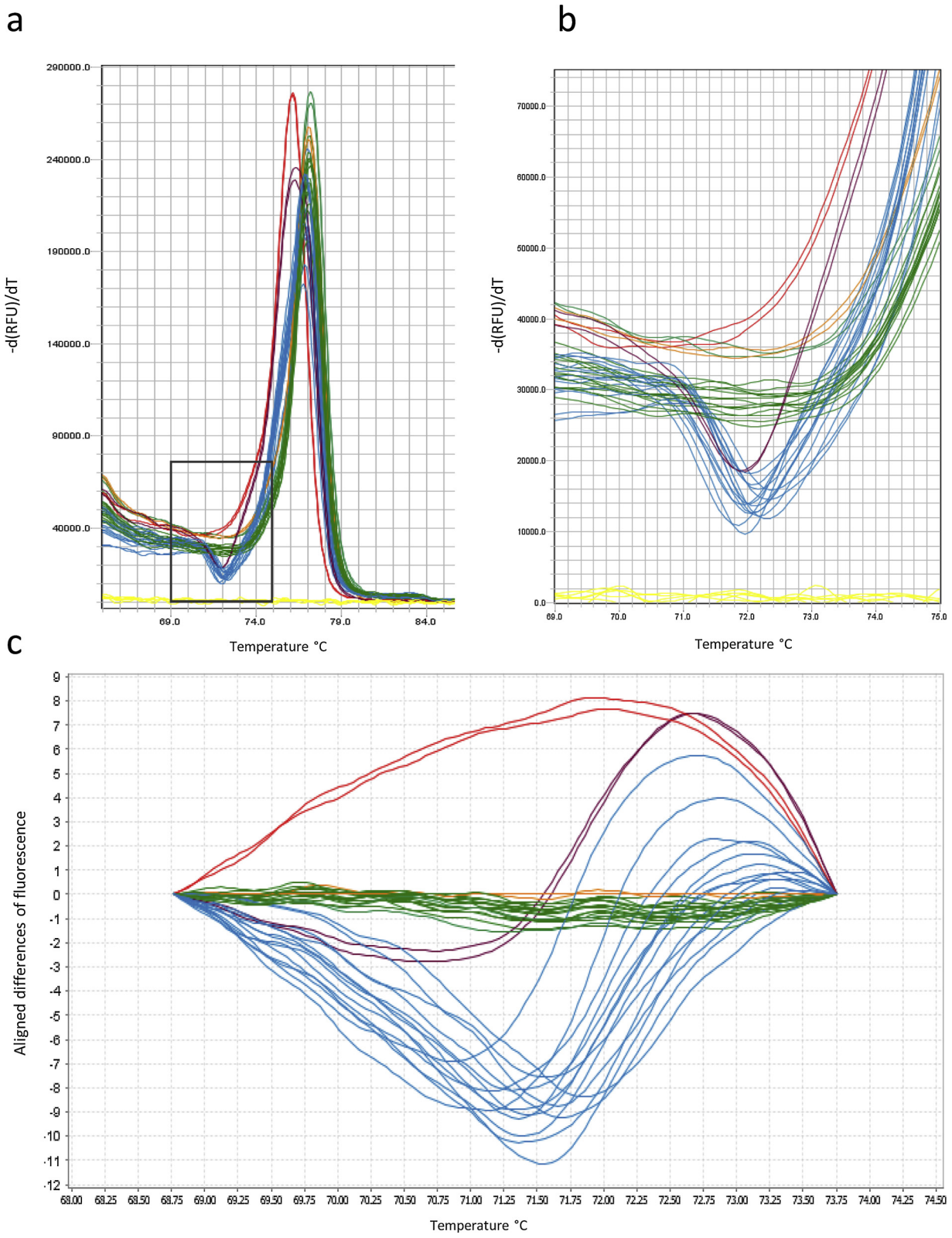


Fig. 4. Melting curves obtained with field samples. a. For the clarity of the figure, only 15 samples from 2017 are shown (of 128 positive samples from 2016 and 2017). Seven samples exhibit a typical drop before the peak (blue) and eigth do not show a drop (green). As positive controls, pure plasmids var1, var2 and a mix (1:1) were included in the experiment (red, orange and purple respectively). The number of plasmid copies for this experiment was 400 for both var1 and var2. b. Magnification of a) showing the dips for some samples. c. Normalized difference fluorescences curves of the step (68.75–73.75 °C) before the main fusion peak; the var2 is used as a reference (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Ct values and ratios of variants found in co-infected fields samples of *A. gueldenstaedtii*. The first number in the name of the sample indicates the year (*i.e.* 15/XX is from 2015). The tissues sampled are indicated in superscript: g, gills; f, fins; gf, pool of gills and fins. The farm (Fx) of origin is indicated in brackets. The columns var1 and var2 indicate the numbers of clones obtained after cloning and sequencing of the PCR products.

Sample	Ct (PCR Taqman)	Ct (PCR HRM)	var1	var2	var1/ var1 + var2 (%)
15/052^{g} (F2)	nd	22.91	2	1	67
16/85^{gf} (F1)	28.08	26.18	1	7	13
17/113-2^{f} (F3)	29.8	31,14	1	18	5
17/284-5^{f} (F2)	24.4	24,51	6	9	40
17/285-1^{g} (F1)	32.1	33.08	4	7	36
17/286-1^{g} (F1)	28.5	29.85	1	10	9
17/286-2^{f} (F1)	26.5	27.42	1	8	11
17/302-5^{f} (F2)	29.7	30.39	4	20	17
17/381-6^{f} (F2)	29.2	29.73	1	14	7

a diagnostic method, although this was not the initial goal. However, the method should be also tested on host species other than Russian sturgeon to ensure the absence of non-specific products in different genetic backgrounds. Furthermore, a slight shift in the Tm value was observed at low plasmid loads. This shift observed for low loads of DNA had been previously reported (Yu et al., 2015). General recommendations of both thermocycler manufacturers include testing samples at Ct < 30. PCR-HRM is therefore better adapted to genotyping samples with moderate to high viral loads, for instance a collection of samples previously designated positive with TaqMan PCR.

A variation of 1 °C was observed between the Tm values of the two alleles differing by five substitutions. It was therefore possible to distinguish different amplicons and theoretically both viral variants of AcIV-E. Comparatively, a difference of 1 °C was also observed in two human herpesviruses, HSV-1 and HSV-2, differing by eight mutations in the amplified region (Lieveld et al., 2017). However, for the HSV and AciV-E models, it is not possible to separate the melting peaks of the two genotypes in mixture. In AcIV-E, a unique peak with an intermediate Tm was observed when using equal quantities of plasmids. The impossibility of obtaining two distinct peaks is likely due to the weak difference (1 °C) between the Tm values.

In our study, the Tm values varied with the thermocycler used (CFX96 or QS5), although a difference of 1 °C between var1 and var2 was consistently obtained. We have no explanation for this thermocycler effect, but it has already been observed in another study using four different HRM thermocyclers targeting human poxviruses (Gelaye et al., 2017). The influence of the thermocycler on Tm further stresses the importance of using reference controls (recombinant plasmids or ultramers, for instance) in each assay.

Using recombinant plasmids in order to mimic viral genomes, we evaluated the efficiency of the assay to identify both variants in a single reaction. At ratios favoring one variant (1:5 to 1:80), the Tm was similar to that of the dominant allele and was therefore uninformative as to the presence of the minor allele. However, for allele ratios of between 1:5 and 1:40, we attributed the presence of a dip in the melting curve to the presence of heteroduplexes melting prematurely, thus indirectly indicating the presence of the minor variant. The detection of heteroduplexes has been used in other studies to differentiate homozygote and heterozygote genomes in eukaryotes or to screen for atypical viral strains (Sabol et al., 2009; Tajiri-Utagawa et al., 2009; Wittwer, 2009). For instance, distinct viral subtypes of the influenza virus were identified by mixing PCR products of an unknown isolate with PCR products of a reference strain (Lin et al., 2008).

Out of 128 virus-positive field samples analyzed using PCR-HRM, var2 was the most frequently found, alone or occasionally mixed with var1 as confirmed by sequencing. Interestingly, var1 was never found alone. The apparent absence of samples with var1 alone or dominant, may be due to the limited sampling. In the present work, samples were only from one host species (Russian sturgeon) and from three farms in one country (France). Other fish species and other regions in Europe should be screened. For example, the var1 allele has previously been detected, in co-infection with var2, in 2015 in another European country and in another sturgeon species (*Huso huso*) in 2012 (Bigarré et al., 2017). Alternatively, the low occurrence of var1 may arise from low replication efficiency of this variant or from near-elimination by the fish immune system when this variant is present alone. Possibly, var1 may replicate at detectable levels only in mixed infections due to stimulating factors provided by var2.

The PCR-HRM assay proved to be useful for screening large numbers of sturgeon samples at a reduced cost compared with sequencing. The method is specific to AcIV-E and does not target other NCLDVs. Therefore, to diagnosis any European sample of unknown status, we recommend first using the TaqMan real-time PCR protocols available for AcIV-E and NV (Bigarré et al., 2017; Clouthier et al., 2015). Subsequently, PCR-HRM can be used specifically on samples that prove to be AcIV-E-positive. PCR-HRM can nevertheless be used in first-intention diagnosis, but it is important to keep in mind that the Tm deviates slightly at low concentrations. For genotyping, PCR-HRM can be used on a large scale in Europe to screen AcIV-E-positive Russian sturgeon – a species which is very sensitive to the virus – but also other sturgeon species of different geographic origins. PCR-HRM will be useful to evaluate the prevalence of the two AcIV-E variants in Europe. In particular, it is of interest to collate data on var1 epidemiology, including its biological impact on its host. Wide screening should shed light on the origins of var1 and var2, and determine if they have both been imported into Europe *via* commercial trade or if they diverged in Europe from an endemic virus or from a recent imported ancestor.

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Ethical approval

The samples originated from moribund or dead farmed fish that had not undergone experimentation and that had been euthanized in accordance with animal welfare ethics.

Conflict of interest

B. Debeuf and V. Chesneau work for private companies cited in the authors' list. The other authors declare that they have no competing interests regarding the publication of this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.12.006.

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