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Short Communication

Chilean IPNV isolates: Robustness analysis of PCR detection

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ABSTRACT

Background: The genomes of several infectious pancreatic necrosis viruses (IPNVs) isolated in Chile were sequenced with a single amplification approach for both segments A and B. The resulting sequences were then used to determine the conservation of the primer-binding regions used in polymerase chain reaction (PCR)-based diagnostic methods proposed in the literature. Thus, the robustness of each technique was studied, particularly the eventual effect of further mutations within the primer-binding sites.
Results: On analysis, most methods currently used to detect Chilean IPNV varieties were deemed adequate. However, the primers were designed to be genogroup specific, implying that most detection methods pose some risk of detecting all strains prevalent in the country, due to the coexistence of genogroups 1 and 5.
Conclusions: Negative results must be interpreted carefully given the high genomic variability of IPNVs. Detection techniques (quantitative reverse transcription (qRT)-PCR) based on degenerate primers can be used to minimize the possibilities of false-negative detections.

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1. Introduction

Infectious pancreatic necrosis virus (IPNV) belongs to the *Birnaviridae* family and the *Aquabirnavirus* genus. It is composed of an unenveloped icosahedral capsid and a bisegmented genome with double-stranded RNA (dsRNA).

The smaller segment, segment B (2784 bp), contains a single open reading frame (ORF) encoding for the VP1 protein, a dsRNA-dependent RNA polymerase. The longer segment, segment A (3097 bp), contains two ORFs. The larger ORF encodes for a 106-kDa polyprotein that is cleaved cotranslationally by the nonstructural protease VP4, hence generating mature pre-VP2 and VP3 (the major and minor capsid proteins, respectively), and VP4. The sequence of this ORF has been used to classify Aquabirnaviruses into 6 distinct genogroups by their geographical origin. The smaller ORF encodes for a 17-kDa nonstructural protein of unknown function [1].

IPNV has been reported to cause a highly contagious disease in fish less than 4 months old, leading to high mortality rates. Moreover, survivors may become lifelong asymptomatic carriers of the disease, thus acting as virus reservoirs [2].

Salmonids are not endemic to Chile, having been introduced for sport fishing in 1905. However, salmon aquaculture grew into a commercial market only in the 1980s. With the rapid growth of its salmonid aquaculture industry, Chile has become the second largest exporter of salmon worldwide in the past two decades [3].

The rapid growth of this industry led to a local demand for the import of eggs. Thus, salmon pathogens were introduced [4]; IPNV was first isolated and characterized in Chile in 1984 [5], [6]. IPNV was not introduced in a singular event, and the current virus isolates from local salmon indicate both European and North American origins [7], [8], [9], [10].

Pathogen introduction and diversification continues to hinder disease detection. Therefore, diagnostic methods that can detect a wide range of IPNV strains are needed. To assess the efficacy of the current detection methods in detecting a wide genetic range of IPNV, several Chilean isolates were sequenced and primer-binding areas were compared with their respective primers.

2. Material and methods

2.1. Fish samples and screening of IPNV samples

Samples of Atlantic salmon (*Salmo salar*) and Coho salmon (*Oncorhynchus kisutch*) fry were collected from several freshwater and seawater farms in southern Chile during the years of suspected outbreaks of the disease: 2010, 2012, and early 2013. Only fish with

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characteristic symptoms of the disease were collected. For each individual fry, the kidney and spleen extracts were pooled and then split into samples for cell culture infection and viral RNA analysis.

The samples for cell culture were stored in L-15 (Leibovitz) medium supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) and 50 μg mL^{-1} gentamicin. Confluent monolayers of Chinook salmon (*Oncorhynchus tshawytscha*) embryonic cells (*CHSE-214*) were used for IPNV propagation and the immunofluorescence assay [11].

The samples for viral RNA analysis were stored in 95% ethanol and subsequently extracted with the EZNA Total RNA Kit (Omega Biotek, Norcross, GA) according to the manufacturer's instructions. These samples were then used in separate quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses for both segments. To detect segment A, SYBR Green analysis was conducted using a primer set of WB1 and WB2. To detect segment B, a TaqMan probe approach was used, using a primer set of VP1f (GTTGATMMASTACACCGGAG) and VP1r (AGGTCHCKTATGAAGGAGTC), and the probe VP1(TACATA GGCAAAACCAAA) [7].

2.2. Genogrouping IPNV isolates

IPNV-positive samples, as determined by the immunofluorescence and/or quantitative PCR assays, were amplified for sequencing using a PCR Multigene Labnet device (Edison, NJ, USA). To allow for the detection of a wide range of strains with high-depth sequencing, most samples were initially sequenced on the 1180-bp segment A, corresponding to the VP2 gene. This was achieved using the primers A1 F and A2 R [12] at a final concentration of 0.5 μM in 15 μL of 2× DreamTaqTM Green PCR Master Mix (Fermentas, Vilnius, Lithuania), 0.8 μL of MMLV Reverse Transcriptase and RNase Block (Stratagene, La Jolla, CA, USA), and 8.2 μL of RNase-free water in a reaction volume of 30 μL.

The amplified PCR products were purified using an E.Z.N.A. Cycle-Pure Kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's instructions. The purified complementary DNA (cDNA) was eluted and then sequenced by Macrogen Inc. (Seoul, Korea) using an ABI3730XL DNA Analyzer (Life Technologies, Carlsbad, CA, USA).

The resulting sequences were analyzed using BioEdit, version 7.1.9 [13], and aligned with previously reported sequences of VP2 aquatic birnaviruses [14] and locally isolated IPNVs [7], [8], [9].

From a total of 54 partially sequenced IPNV isolates, 26 IPNV isolates were selected for the primer binding analysis. Closely related sequences were excluded to maximize diversity in the analysis.

2.3. Sequencing strategy

The viral isolates selected for primer binding analysis were amplified by PCR using the primers A-A5EJ′NC and A-IPNVEJ R for segment A and B-B5EJ′NC and B-IPNVEJ R for segment B.

The forward primer of segment A was derived from the 1998 study by Yao and Vakharia [15]. The corresponding reverse primer was designed based on several IPNV sequences from genogroups I, III, and V, and MABV available in GenBank (accession numbers AF078668. 1, NC_001915.1, AY283780.1, AM-98, AY780921.3, AY780924.3, D26526.1, AY780919.1, AJ622822.1, AY354520.1, AY354521.1, AY379738.1, AY379740.1, AY379742.1, AY823632.1, AY283783.1, and AY283785.1).

Similarly, the forward primer of segment B was based on the design of Yao and Vakharia in 1998 [15]. The corresponding reverse primer was based on IPNV sequences available in GenBank (accession numbers AF078669.1, AY780928.1, D26527.1, EU665685.1, NC_001916.1, AY780926.1, AY780931.1, AJ622823.1, AY354522.1, AY354523.1, AY354524.1, AY379739.1, AY379741.1, AY379743.1, AY123970.1, and AY129665.1).

To amplify both segments A and B, 3.0 μL of viral RNA was mixed with the primers A-A5EJ′NC and A-IPNVEJ R for segment A and B-B5EJ′NC and B-IPNVEJ R for segment B to a final concentration of 0.5 μM in 15 μL of 2X DreamTaqTM Green PCR Master Mix (Fermentas, Vilnius, Lithuania), 0.8 μL of MMLV Reverse Transcriptase and RNase Block (Stratagene, La Jolla, CA, USA), and 8.2 μL of RNase-free water in a reaction volume of 30 μL. The primers used are shown in Table 1.

The RT-PCR amplification profile was as follows: reverse transcription at 50°C for 30 min, pre-denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing temperature of 53.0°C for 30 s for segment A and 57.6°C for segment B, extension at 72°C for 4 min, and a final extension at 72°C for 10 min.

The amplified products were resolved by agarose gel electrophoresis, and the obtained PCR products were purified using an E.Z.N.A. Cycle-Pure Kit (Omega Bio-tek, Norcross, GA, USA), according to the manufacturer's instructions. Semiconductor sequencing was performed on the purified genetic material at OMICS Solutions (Santiago, Chile), using an Ion Torrent™ Personal Genome Machine® (PGM™) with an Ion 316™ chip (Life Technologies, Carlsbad, CA, USA).

The resulting sequences were assembled using the CodonCode Aligner software [16] (version 4.1.1, CodonCode Corporation, Dedham, MA, USA). The assembled sequences were then edited and compared using BioEdit version 7.1.9 [13].

2.4. Mismatch Tm analysis

To detect any variability in the regions used for PCR detection, the resulting sequences were contrasted with several primer sequences previously used to detect IPNV [7], [9], [10], [17], [18], [19], [20].

Mismatch melting temperature analysis was performed for each primer and its binding region using the nearest-neighbor method [21]. In addition, the individual mutations found in the sequenced data, the frequency of each mutation, and the calculated Tm between the primer-binding sites and the respective primer were determined.

3. Results

3.1. Genogrouping of selected Chilean isolates

The selected Chilean isolates were clustered into genogroups 1 and 5, based on the North American and European origins of segment A, as shown in Table 2. The same clustering pattern was observed in the case of segment B sequences (data not shown), and in the traditional clustering method with VP2 amino acid sequences [14].

3.2. Mismatch Tm analysis

The following primer sets (shown as the set's forward primer and then reverse primer) were analyzed, the results of which are shown in Table 3.

The primer sets WB117 (WB117F and WB117R) and SP8 (Sp8F and Sp8R), designed by Calleja [9] to specifically amplify samples of genogroups 1 or 5, respectively, were used to evaluate primer robustness. The WB117 primer set showed only minor differences in 9 samples from genogroup 1, whereas the remaining 17 samples of genogroup 5 showed 8 polymorphisms compared with the primer's target. Thus, the calculated Tm for these pairs was much lower, approximately 30°C for both forward and reverse primers, which indicated a considerably weaker binding and in turn the experimental genogroup specificity stated in the original publication.

Similarly, the primer set Sp8 (Sp8F and Sp8R) showed conserved primer-binding regions for the 17 samples from genogroup 5. Unlike the WB117 set, only few changes were seen in the forward primer-binding region in the other samples, but their effect on the change in melting temperature was greater. Conversely, the reverse primer was located in a well-conserved area overall, with notable mutations for 1 of the sequenced samples, specifically the VUV/84 strain.

Table 1
Primers used for IPNV segment amplification.

Primer name	Sequence (5′ → 3′)	Tm (°C)	%GC	Amplicon (bp)	Segment
A-A5EJ'NC	GGAAAGAGAGTTTCAACG	55.3	44.44	3062	A
A-IPNVEJ R	ATCAGTCAGGAAAGAGAG	51.8	44.44		
B-B5EJ'NC	GGAAACAGTGGGTCAACG	62.2	55.56	2761	B
B-IPNVEJ R	TTTTCAGGGGTCATGTTGG	63.8	47.37		

Local laboratories, including our own, use primer sets such as WB (WB1 and WB2) [19] and VP2 (VP2F and VP2R) [10] to detect and quantify IPNV RNA. The WB primer set showed polymorphisms in all samples, particularly in those from genogroup 5, presenting more than 2 changes within each binding area. However, the VP2 set was conserved to a greater extent. The forward primer-binding area showed few polymorphisms in 8 of the samples from genogroup 1. The reverse primer did not show any polymorphisms in 23 of the analyzed samples. However, the sample VUV/84 presented 3 polymorphisms in the reverse primer-binding area, leading to a decrease of more than 30°C in its Tm when compared to a perfect fit.

The primer sets designed by McBeath (McBeathF and McBeathR) [18], Bowers (Bowers 1916 and Bowers 1999) [17], and Taksdal (TaksdalF and TaksdalR) [20] primer sets are described in the literature and used in IPNV diagnosis. In McBeath's study, the primer set showed conserved binding regions for 17 strains from genogroup 5 for the forward primer and 16 for the reverse primer. Genogroup 1 strains showed at least 3 polymorphisms for both the forward and reverse primers.

On the contrary, Bowers' primers [17] presented conserved binding regions for genogroup 1 samples, whereas the reverse primer-binding region was completely conserved in only 1 sample, and the remaining genogroup 1 samples only presented 1 polymorphism. Samples from genogroup 5 showed 4 or more differences, in both forward and reverse primers, between the corresponding binding region and the primer's sequence.

Unlike previous examples, Taksdal's primer set [20] contained highly conserved primer-binding regions. The forward primer had an identical sequence to its binding region for genogroup 5 samples, and only 1 polymorphism for most genogroup 1 strains, except for 1 sample. In comparison, the reverse primer is located in a more conserved area, which is identical in all samples, except in one that differed in 2 bases.

Finally, the VP1 primer set (VP1F and VP1R) [7] was analyzed for its use in our laboratory to detect segment B of IPNVs using a TaqMan assay. In order to detect most, if not all, IPNV strains, the forward and reverse primers were designed as degenerated primers around a fully conserved area of the viral genome used as the TaqMan probe target. As degenerated primers were used, the primer sequence closest to the binding region sequence was considered for the analysis. The binding area for genogroup 5 was found to be completely conserved. Similarly, genogroup 1 was also conserved, except for the VUV/84 strain, the only genogroup 1 sample not directly related to the West Buxton strain, which had 1 polymorphism in the binding areas of both the forward and reverse primers.

Table 2
IPNV strains sequenced in this study.

Strain	Host	Genogroup
VUV/84	Rainbow trout	1
23459	Coho salmon	1
24555	Coho salmon	1
25227	Coho salmon	1
25347	Coho salmon	1
IPNV11	Coho salmon	1
ABCD1	Coho salmon	1
LKJH6	Coho salmon	1
PITR1C	Coho salmon	1
24315	Atlantic salmon	5
24548	Atlantic salmon	5
24655	Atlantic salmon	5
25237	Coho salmon	5
ALKA2	Atlantic salmon	5
ALKA3	Atlantic salmon	5
BLCO3	Atlantic salmon	5
HP1	Atlantic salmon	5
KJKB3	Atlantic salmon	5
EBPS1	Atlantic salmon	5
EBPS2	Atlantic salmon	5
MPMA1	Atlantic salmon	5
IPNV3	Atlantic salmon	5
CGPC1	Atlantic salmon	5
CGPC5	Atlantic salmon	5
CNJJ2	Atlantic salmon	5
RV04	Atlantic salmon	5

4. Discussion

The results of using the primer sets WB117 and Sp8 [9], a set of primers designed to distinguish between genogroups 1 and 5, were expected: the marked decrease in the melting temperature between the primer and its binding region inhibited cross-genogroup amplification. In fact, in the primer set WB117, both the forward and reverse primers caused a decrease of about 30°C when matched with non-corresponding genogroups. However, this phenomenon is not as clear for primer set Sp8, wherein only the forward primer (Sp8F) showed unexpected differences in melting temperature between genogroups. By contrast, the reverse primer (Sp8R) showed polymorphisms and differences in melting temperature in only 1 strain. Nevertheless, as exponential amplification depends on both primers, Sp8 remains genogroup specific, and its selectivity depends only on the forward primer. Thus, Sp8R can be easily used with a different forward primer to detect both genogroups. It is worth noting that the use of probes are included in the complete protocol [8], which further increase specificity.

Of the analyzed primer sets described in the literature, and although not indicated specifically in the corresponding studies, the sets designed by McBeath [18] and Bowers [17] tend to be specific for genogroups 5 and 1, respectively. By contrast, the primer set designed by Taksdal [20] is highly conserved between genogroups 1 and 5, and can thus be used for regular IPNV screening analysis in Chilean fish, with coexistence of both IPNV genogroups.

Of the primers used in this study, the WB [19] set was slightly more specific for genogroup 1. However, this set is thermodynamically stable and the melting temperature of both primers and their binding regions remains above 65°C despite multiple polymorphism, due to the length of the primers. The VP2 primer set showed a similar pattern, with generally stable melting temperatures, remaining above 60°C for all strains except VUV/84. When tested in our laboratory, this primer set could detect the VUV/84 strain, but at a higher threshold cycle than the WB set (data not shown). This may have at least one negative implication, as lower concentrations of certain viral strains might not be detected by the VP2 primer set but by the WB primer set.

In addition, although the Taksdal [20], WB [19], and VP2 [10] primer sets can be used to detect isolated IPNV samples analyzed in this study, the high variability of IPNVs is worth noting, wherein several

Table 3
Mismatch Tm analysis for multiple primers and the sequences of 26 selected strains for the primer binding. Underlined bases in bold indicate the differences between the primer and the binding site sequences found in the analyzed strains.

WB117 F primer sequence

Strains	Groups	GCGGTTCGACTTCATTCTACA	Diff	Tm
8	1	GCGGTTCGACTTCAT**C**CTACA	1	67.2
1	1	**A**CGGTTCGACTTCATTCTACA	1	67.2
17	5	**CAA**GTTCGACTTC**CAG**CT**GG**A	8	33.3

WB117 R primer sequence

Strains	Groups	GAGCTTGTCACGGAGACCAC	Diff	Tm
8	1	GAGCTTGTCACGGAGACCAC	0	72.5
1	1	GAGCTTGTCACGG**TA**ACCAC	2	66.1
17	5	GAGCT**GAC**CAC**T**G**T**GAC**A**AC	6	30.7

SP8 F primer sequence

Strains	Groups	CTGAACGGGACGCTCAAC	Diff	Tm
17	5	CTGAACGGGACGCTCAAC	0	70.9
8	1	CT**C**AACGGGAC**C**CT**G**AA**T**	4	38.4
1	1	CT**C**AA**T**GGGAC**C**CT**G**AAC	4	24.2

SP8 R primer sequence

Strains	Groups	TCAGGCTCTCCACCTCAGAC	Diff	Tm
16	5	TCAGGCTCTCCACCTCAGAC	0	72.9
8	1	TCAGGCTCTCCACCTCAGA**A**	1	69.8
1	5	TCAGGCTCTCCACCTC**G**GAC	1	66.3
1	1	T**T**AGGCTCTC**T**AC**T**TCAGAC	3	41.1

WB1(F) primer sequence

Strains	Groups	CCGCAACTTACTTGAGATCCATTATGC	Diff	Tm
5	1	CCGCAAC**C**TACTTGAGATCCATTATGC	1	70.1
2	1	CCGCAACTTAC**C**TGAGATCCATTATGC	1	70.1
1	1	CCGCAACTTAC**C**TGAGATCCAT**C**ATGC	2	67.9
1	1	CCGCAAC**C**TACTT**A**AGATCCATTATGC	2	65.8
17	5	CCGCAACTTAC**C**TGA**A**ATCCATTATGC	2	65.6

WB2(R) primer sequence

Strains	Groups	CGTCTGGTTCAGATTCCACCTGTAGTG	Diff	Tm
3	1	CGTCTGGTTCAGATTCCACCTGTAGTG	0	75.0
1	1	CGTCTGGTTCAGATTCCACCT**A**TAGTG	1	71.4
5	1	CGTCTGGTTC**G**GATTCCACCTGTAGTG	1	70.5
1	5	CGTCTGGTTCA**C**ATTCCA**T**CTGTAGTG	2	67.7
16	5	CGTCTGGTTC**GC**ATTCCA**T**CTGTAGTG	3	65.0

VP2 F primer sequence

Strains	Groups	TCCAACTACGAGCTGATCCC	Diff	Tm
18	1.5	TCCAACTACGAGCTGATCCC	0	71.0
7	1	TCCAACTA**T**GAGCTGATCCC	1	63.5
1	1	TCCAACTA**T**GAGC**C**GATCCC	2	59.8

VP2 R primer sequence

Strains	Groups	GTCCTCTCCTTGTACTCCTC	Diff	Tm
23	1.5	GTCCTCTCCTTGTACTCCTC	0	68.1
1	1	GTCCTCTCCTT**A**TACTCCTC	1	62.5
1	5	GTCCT**T**TCCTTGTACTCCTC	1	61.0
1	1	GTCCT**T**TC**T**TTGTA**T**TCCTC	3	36.7

McBeath F primer sequence

Strains	Groups	GCCAAGATGACCCAGTCCAT	Diff	Tm
17	5	GCCAAGATGACCCAGTCCAT	0	72.1
1	1	GCCAAG**TTC**ACCCAGTC**A**AT	3	49.9
8	1	GCCAAG**TTT**AC**G**CAGTC**A**AT	4	32.0

McBeath R primer sequence

Strains	Groups	TGACAGCTTGACCCTGGTGAT	Diff	Tm
16	5	TGACAGCTTGACCCTGGTGAT	0	73.4
1	5	TGACAGCTTGACCCT**T**GTGAT	1	67.9
1	1	**G**G**C**CAGCTTGACCC**GT**GTGAT	4	64.4
1	1	**A**G**C**CAGC**C**TGACCCT**T**GTGAT	4	57.8
4	1	**A**G**C**CAGC**C**TGACCCT**T**GT**A**AT	5	48.2
3	1	**G**G**C**CAGC**C**TGACCCT**T**GT**A**AT	5	46.1

Table 3 (*continued*)

Bowers 1916(F) primer sequence

Strains	Groups	AGGAGATGACATGTGCTACACCG	Diff	Tm
9	1	AGGAGATGACATGTGCTACACCG	0	73.4
17	5	**G**GGAGA**CA**ACATGTGCTACAC**T**G	4	51.1

Bowers 1999(R) primer sequence

Strains	Groups	CCAGCGAATATTTTCTCCACCA	Diff	Tm
1	1	CCAGCGAATATTTTCTCCACCA	0	70.1
8	1	CCAGCGAATATTTTCTCCAC**T**A	1	62.5
2	5	CCAGCGAA**G**AT**C**TTCTC**G**AC**T**A	4	36.9
1	5	CCAGC**A**AA**G**AT**C**TTCTC**T**AC**T**A	5	25.2
14	5	CCAGC**A**AA**G**AT**C**TTCTC**G**AC**T**A	5	25.0

Taksdal F primer sequence

Strains	Groups	ATCTGCGGTGTAGACATCAAAG	Diff	Tm
17	5	ATCTGCGGTGTAGACATCAAAG	0	69.7
8	1	ATCTGCGG**A**GTAGACATCAAAG	1	64.4
1	1	ATCTGCGG**A**GT**G**GACATCAAAG	2	59.2

Taksdal R primer sequence

Strains	Groups	TGCAGTTCCTCGTCCATCCC	Diff	Tm
25	1.5	TGCAGTTCCTCGTCCATCCC	0	74.1
1	1	TGCAGTTC**TG**CGTCCATCCC	2	65.4

VP1 F primer sequence

Strains	Groups	GTTGATMMASTACACCGGAG	Diff	Tm
8	1	GTTGATCAACTACACCGGAG	0	67.7
17	5	GTTGATACAGTACACCGGAG	0	67.1
1	1	GTTGATCAACTACA**A**CGGAG	1	59.6

VP1 R primer sequence

Strains	Groups	AGGTCHCKTATGAAGGAGTC	Diff	Tm
8	1	AGGTCACGTATGAAGGAGTC	0	68.4
17	5	AGGTCCCTTATGAAGGAGTC	0	67.9
1	1	AGGTCACGTATGAA**A**GAGTC	1	62.5

synonymous sites are polymorphic and none of the genomic areas is completely conserved via IPNV evolution. It is also worth noting that, given the size of the Chilean aquaculture, eventual mutations might weaken the efficacy of PCR detection in the future. In fact, one of the studied strains, VUV/84, which has been isolated and grown in cell culture for more than 30 years, showed mutations in the primer-binding sites not seen in other IPNV strains. In practice, the current diagnostic methods can fail to detect and can thus select some undetectable viral strains, although the biosafety regulations for salmon farming call for excluding fish lines infected with IPNV. In this respect, shorter primers, such as the VP2 [10], McBeath [18], and Bowers [17] primer sets, and probes pose a greater risk. Furthermore, as diagnostic methods are used on a daily basis in Chile, using two separate detection methods is impractical. As seen here, the use of longer primers adds robustness to detection assays. Thus, when applied appropriately, the WB primer set can be used for continued IPNV diagnosis, as the eventual changes in its thermodynamic properties are counteracted by the larger primer sizes.

The design of longer primers can be challenging in the case of IPNV, given the high mutation rate of these viruses [22], [23], and the lack of appropriate sites for primer design. Thus, degenerate primers can be considered a viable option for adding robustness. Indeed, the VP1 primer set [7] was designed to bind areas partially conserved in most genogroups using degeneracies to compensate for polymorphic sites. The use of degenerate primers clearly introduces new risks, eventually diminishing PCR specificity [24]. Nevertheless, this growing industry seeks increasingly robust detection methods for a pathology with unknown future variation.

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Conflict of interest

The authors declare no conflict of interest.

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References

[1] Dobos P, Roberts TE. The molecular biology of infectious pancreatic necrosis virus: A review. Can J Microbiol 1983;29:277–384. http://dx.doi.org/10.1139/m83-062.
[2] Julin K, Johansen LH, Sommer AI, Jørgensen JB. Persistent infections with infectious pancreatic necrosis virus (IPNV) of different virulence in Atlantic salmon, *Salmo salar* L. J Fish Dis 2014;38:1005–19. http://dx.doi.org/10.1111/jfd.12317.
[3] Katz J. Salmon farming in Chile. In: Chandra V, editor. Technology, adaptation, and exports: How some developing countries got it right. Washington: World Bank; 2006. p. 193–223.
[4] Bovo G, Håstein T, Hill B, Lapatra S, Michel C, Olesen NJ, et al. QLK2-CT-2002-01546: Fish egg trade work package 1 report: Hazard identification for vertical transfer of fish disease agents. Oslo, Norway: VESO; 2005 1–35(Available at http://www.eurl-fish.eu/~/media/Sites/EURL-FISH/english/activities/scientific%20reports/fisheggtrade%20wp_1.ashx?la = da).
[5] McAllister PE, Reyes X. Infectious pancreatic necrosis virus: Isolation from rainbow trout *Salmo gairdneri* Richardson, imported into Chile. J Fish Dis 1984;7:319–22. http://dx.doi.org/10.1111/j.1365-2761.1984.tb00938.x.
[6] Espinoza E, Farias G, Soler M, Kuznar J. Identity between infectious pancreatic necrosis virus VR-299 and a Chilean isolate. Intervirology 1985;24:58–60. http://dx.doi.org/10.1159/000149619.
[7] Eissler Y, Pavlov MS, Conejeros P, Espinoza JC, Kuznar J. Detection and quantification of Chilean strains of infectious pancreatic necrosis virus by real-time RT-PCR assays using segment B as a target. Lat Am J Aquat Res 2011;39:544–52. http://dx.doi.org/10.3856/vol39-issue3-fulltext-14.
[8] Mutoloki S, Evensen Ø. Sequence similarities of the capsid gene of Chilean and European isolates of infectious pancreatic necrosis virus point towards a common origin. J Gen Virol 2011;92:1721–6. http://dx.doi.org/10.1099/vir.0.030270-0.
[9] Calleja F, Godoy MG, Cárcamo JG, Bandín I, Yánez AJ, Dopazo CP, et al. Use of reverse transcription-real time polymerase chain reaction (real time RT-PCR) assays with Universal Probe Library (UPL) probes for the detection and genotyping of infectious pancreatic necrosis virus strains isolated in Chile. J Virol Methods 2012;183:80–5. http://dx.doi.org/10.1016/j.jviromet.2012.03.022.
[10] Tapia D, Eissler Y, Torres P, Jorquera E, Espinoza JC, Kuznar J. Detection and phylogenetic analysis of infectious pancreatic necrosis virus in Chile. Dis Aquat Organ 2015;116:173–84. http://dx.doi.org/10.3354/dao02912.
[11] Espinoza JC, Cisternas C, Cifuentes F, Enriquez R, Kuznar J. Evaluación de dos métodos de diagnóstico para el virus IPN empleando anticuerpos monoclonales. Acuicultura en Latinoamérica. In: Silva A, Merino G, editors. IX Congreso Latinoamericano de Acuicultura. 2° Simposio de Avances y perspectivas de la Acuicultura en Chile. Coquimbo, Chile: Universidad Católica del Norte, Asociación Latinoamericana de Acuicultura; 1996. p. 276–9.
[12] Blake SL, Schill WB, Mcallister PE, Lee MK, Singer JT, Nicholson BL. Detection and identification of aquatic birnaviruses by PCR Assay. J Clin Microbiol 1995;33:835–9.
[13] Hall TA. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp 1999;41:95–8.
[14] Blake S, Ma JY, Caporale D, Jairath S, Nicholson B. Phylogenetic relationships of aquatic birnaviruses based on deduced amino acid sequences of genome segment A cDNA. Dis Aquat Organ 2001;45:89–102. http://dx.doi.org/10.3354/dao045089.
[15] Yao K, Vakharia V. Generation of infectious pancreatic necrosis virus from cloned cDNA. J Virol 1998;72:8913–20.
[16] CodonCode Aligner version 4.1.1 made by CodonCode Corporation. Available at http://www.codoncode.com/.
[17] Bowers RM, Dhar AK. Effect of template on generating a standard curve for absolute quantification of an RNA virus by real-time reverse transcriptase-polymerase chain reaction. Mol Cell Probes 2011;25:60–4. http://dx.doi.org/10.1016/j.mcp.2010.12.002.
[18] McBeath AJA, Snow M, Secombes CJ, Ellis AE, Collet B. Expression kinetics of interferon and interferon-induced genes in Atlantic salmon (*Salmo salar*) following infection with infectious pancreatic necrosis virus and infectious salmon anaemia virus. Fish Shellfish Immunol 2007;22:230–41. http://dx.doi.org/10.1016/j.fsi.2006.05.004.
[19] Williams K, Blake S, Sweeney A, Singer JT, Nicholson BL. Multiplex reverse transcriptase PCR assay for simultaneous detection of three fish viruses. J Clin Microbiol 1999;37:4139–41.
[20] Taksdal T, Dannevig BH, Rimstad E. Detection of infectious pancreatic necrosis (IPN) virus in experimentally infected Atlantic salmon parr by RT-PCR and cell culture isolation. Fish Pathol 2001;21:214–8.
[21] SantaLucia J, Hicks D. The thermodynamics of DNA structural motifs. Annu Rev Biophys Biomol Struct 2001;33:415–40. http://dx.doi.org/10.1146/annurev.biophys.32.110601.141800.
[22] Lauring AS, Frydman J, Andino R. The role of mutational robustness in RNA virus evolution. Nat Rev Microbiol 2013;11:327–36. http://dx.doi.org/10.1038/nrmicro3003.
[23] Skjesol A, Skjæveland I, Elnæs M, Timmerhaus G, Fredriksen BN, Jørgensen SM, et al. IPNV with high and low virulence: Host immune responses and viral mutations during infection. Virol J 2011;8:396. http://dx.doi.org/10.1186/1743-422X-8-396.
[24] Linhart C, Shamir R. The degenerate primer design problem: Theory and applications. J Comput Biol 2005;12:431–56. http://dx.doi.org/10.1089/cmb.2005.12.431.