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Development and validation of a new SYBR green-based qPCR assay for the broad detection of *Novirhabdovirus salmonid* (IHNV) genotypes

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ABSTRACT

Infectious Haematopoietic Necrosis Virus (IHNV) is a WOAH-notifiable pathogen causing severe economic losses in global salmonid aquaculture. While the WOAH-recommended TaqMan assay is the current standard, its diagnostic efficacy is frequently compromised by high execution costs and reduced sensitivity toward specific genotypes, particularly the JS lineage, due to single nucleotide polymorphisms (SNPs) in the probe-binding region. To address these limitations, this study developed a novel SYBR Green-based qPCR assay targeting the highly conserved nucleoprotein (N) gene. Meticulous thermodynamics of a new primer set was optimized by balancing GC content, melting temperature (*Tm*), and Gibbs free energy (ΔG)—to ensure broad genotype inclusivity (U, M, E, JN, and JS) while eliminating non-specific amplifications and primer-dimers. The assay demonstrated superior analytical performance, particularly for the JS genotype, yielding a significantly lower Cq value (16.69) compared to the WOAH assay (20.30), which suffered from probe-mismatch issues. The estimated $LoD_{95\%}$ was 16.92 copies/reaction, with 100% diagnostic sensitivity and specificity in experimentally infected samples. Validation using 123 field samples from Republic of Korea and Poland revealed that the new assay identified eight IHNV-positive domestic samples initially missed by the WOAH-recommended method, all of which were subsequently confirmed via sequencing. With a coefficient of variation (CV) consistently below 5% and proven inter-laboratory reproducibility across seven facilities, this assay provides a robust, highly sensitive, and cost-effective alternative to probe-based methodologies. It is suited for large-scale surveillance and routine diagnostics, especially in resource-limited settings where detecting diverse viral mutations is critical.

1. Introduction

Infectious haematopoietic necrosis virus (IHNV) is the etiological agent of Infectious haematopoietic necrosis (IHN), a disease notifiable to the World Organisation for Animal Health (WOAH, 2021; Chapter 2.3.5.). IHNV is a critical viral pathogen that significantly impacts

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salmonid aquaculture, primarily affecting Rainbow trout (*Oncorhynchus mykiss*) farming in North America, Europe, and Asia (Kim et al., 2007; Xu et al., 2019). The global expansion of the salmonid industry relies heavily on the international trade of eyed eggs, which poses a continuous risk for transboundary spreading and the introduction of novel genotypes into immunologically naive fish populations (WOAH, 2021; Chapter 2.3.5.).

IHNV genotypes are geographically classified into five major groups: L, M, and U in North America; E in Europe; and J in Japan and Republic of Korea (Kurath et al., 2003; Enzmann et al., 2005). Within the J genotype, two distinct lineages—JRt Nagano (JN) and JRt Shizuoka (JS)—have been identified, both of which are prevalent in rainbow trout farms in Republic of Korea (Kim et al., 2007; Lim et al., 2021). Since its initial detection in 1991, only the J genotype has been reported in Republic of Korea, causing severe hemorrhagic necrosis and high mortality in juvenile fish (Park et al., 1993; Kim et al., 2007).

Republic of Korea has been importing fertilized salmonid eggs and juveniles from IHNV-endemic countries such as Norway, the United States of America, and Denmark and only pathogen-free ("IHNV/ISAV-free") eyed eggs certified under WOAH standards are permitted for aquaculture use (WOAH, 2019). Current diagnostic standards for IHNV primarily rely on the WOAH-recommended Reverse Transcription quantitative PCR (RT-qPCR) protocol, which utilizes a hydrolysis probe (TaqMan) assay targeting the viral nucleoprotein (N) gene (WOAH, 2021; Chapter 2.3.5.). However, previous studies have described ambiguous amplification patterns in strains such as FV23, FV91–40, and D332–92, including flat amplification curves and weak fluorescence signals that may lead to false-negative interpretations (Hoferer et al., 2019; Purcell et al., 2013).

This indicates that the probe-based method has significant diagnostic limitations, particularly regarding genotype-specific sensitivity. Genetic analysis of the JS genotype reveals a critical single nucleotide polymorphism (SNP) within the probe-binding region originally described by Purcell et al. (2013). Such mismatches, especially at a central position critical for annealing, significantly compromise probe hybridization efficiency, leading to atypical amplification curves, weak fluorescence signals, and an increased risk of false-negative results (Hoferer et al., 2019; Purcell et al., 2013). Furthermore, the prohibitive costs of TaqMan reagents and the requirement for specialized equipment make routine surveillance unsustainable in many diagnostic laboratories (Ponchel et al., 2003; Bohara et al., 2022).

To overcome these challenges, there is an urgent need for a more robust and cost-effective diagnostic alternative. SYBR Green-based qPCR assay offers several advantages, including cost-effectiveness, broader mismatch tolerance, and simplified assay design that is adaptable to both laboratory and field settings (Bustin et al., 2005; Wong and Medrano, 2005). Although SYBR Green chemistry presents inherent challenges, such as potential non-specific amplification and primer-dimer formation due to its non-sequence-specific DNA binding, these issues were minimized through rigorous in silico primer design and systematic assay optimization (Bustin et al., 2005; Wong and Medrano, 2005). The precise balancing of GC content, melting temperature (*Tm*), and Gibbs free energy (ΔG) of primers proved critical for eliminating non-specific amplifications and primer-dimer formation, which are common pitfalls in SYBR Green chemistry, maximizing mismatch tolerance and reaction efficiency (Wong and Medrano, 2005; Chico et al., 2006; Bustin et al., 2005). This approach could ensure the stable detection of diverse IHNV genotypes, including the problematic JS lineage, while eliminating common pitfalls such as non-specific amplification and primer-dimer formation (Purcell et al., 2013; Batts et al., 2022).

The objective of this study was to develop and validate a new SYBR Green-based qPCR assay targeting the conserved regions of the IHNV N gene to enable broad-spectrum detection (U, M, E, JN, and JS). The robustness of the new assay was initially estimated by ensuring adequate primer characteristics including GC content, *Tm*, and ΔG. The performance of the selected primer pairs was then evaluated by comparing their analytical sensitivity (ASe) and analytical specificity (ASp) against the WOAH probe-based RT-qPCR. To ensure reliability, we verified the repeatability and reproducibility of the test method. Furthermore, diagnostic sensitivity (DSe) and diagnostic specificity (DSp) were assessed using samples from experimentally infected fish. Finally, to secure clinical reliability, we analyzed domestic and international field samples to confirm its practicality under field conditions.

2. Materials and methods

2.1. Virus strains and cell lines

The IHNV genotypes used in this study included U (Baker), M (220–90), E (32–87), JN (RtPc22), and JS (RTYK1801) (Table 1). These viruses were propagated in *Epithelioma papulosum cyprini* (EPC) cells at 20 °C in Leibovitz's L-15 medium (Gibco, USA) supplemented with 10% fetal bovine serum (HI FBS, Gibco) and 1% antibiotic–antimycotic (Gibco) (WOAH, 2021, Chapter 2.3.5.). The virus stock from the supernatant was stored at −70 °C until used for diagnostic method development and infection experiments.

2.2. RNA extraction and cDNA synthesis

Viral RNA was extracted from the stored virus supernatant using the High Pure Viral RNA Kit (Roche), and total RNA from IHNV-infected tissues was isolated using the RNeasy Mini Kit (Qiagen, Germany), following the respective manufacturers' protocols. For cDNA synthesis, 3 μg of RNA was reverse transcribed using RevertAid H Minus Reverse Transcriptase (Thermo Scientific, USA) with random hexamers (Thermo Scientific). The synthesized cDNA was stored at −20 °C until further use.

2.3. Primer design and thermodynamic optimization

To ensure broad genotype inclusivity, conserved regions within the IHNV nucleoprotein (N) gene were identified by aligning sequences of all major genotypes (U, M, E, JN and JS) using CLUSTAL 2.1. Primers were meticulously designed using Primer 3 (v.0.4.0, https://primer3.ut.ee/), focusing on thermodynamic optimization to maximize mismatch tolerance especially for the JS genotype and reaction efficiency. The thermodynamic parameters including GC content, *Tm*, and ΔG, were systematically balanced using the OligoAnalyzer™ tool (IDT, https://sg.idtdna.com/pages/tools/oligoanalyzer) to eliminate non-specific amplification, hairpin structures, and primer-dimer formation. The specificity of the optimized primers was further verified via National Center for Biotechnology Information (NCBI) BLAST against salmonid genomes.

2.4. Plasmid construction

To establish standard curves and determine the limit of detection (LoD) for both assays, two types of plasmid DNA (pDNA) standards were constructed: pDNA-S for the new SYBR Green assay and pDNA-W for the WOAH probe-based RT-qPCR. For pDNA-S, a 1019 bp partial fragment of the IHNV N gene was amplified using the IHNV qN 2F and IHNV N Partial 1R primers designed in this study. For pDNA-W, a 106 bp

Table 1
List of viruses used in this study.

Virus	Genotype	Strain	Reference
IHNV	U	Baker	Peñaranda et al., 2011
	M	220–90	Ammayappan et al., 2010
	E	32–87	Johansson et al., 2009
	JN	RtPc22	In this study (Republic of Korea)
	JS	RTYK1801	Lim et al., 2021

fragment corresponding to the WOAH target region was amplified using the IHNV N 796F and IHNV N 875R primers.

The amplified PCR products for both assays were purified and cloned into standard cloning vectors, and their specific sequences were confirmed through base sequence analysis by Macrogen (Republic of Korea). The concentration of the purified pDNA was measured using a spectrophotometer to calculate the exact copy number based on the molecular weight.

2.5. Quantitative PCR conditions

For optimization of the diagnostic assay, candidate primer sets targeting conserved regions of the IHNV N gene were designed and evaluated based on amplification efficiency, Cq value, melting curve specificity, and the absence of non-specific amplification. Five commercial SYBR Green qPCR reagents were compared, and the QuantiTect SYBR Green PCR Kit (Qiagen, Germany) was selected for the final assay because it provided stable amplification without non-specific amplification in the negative controls (Supplementary Fig. 1 and Supplementary Table1). The reaction conditions were further optimized, and 10 pmol of each primer with annealing/extension at 60 °C for 30 s was selected as the final condition (Supplementary Fig. 2 and Supplementary Table2). The final qPCR protocol consisted of 40 cycles and produced a single target-specific melting peak without amplification in the negative controls (Supplementary Fig. 3 and Supplementary Table 3).

SYBR Green assay reactions were performed using QuantiTect SYBR Green PCR Kit (Qiagen), with 10 pmol of forward/reverse primers and 2 μL of cDNA in a 25 μL total volume. Thermal cycling involved initial denaturation at 95 °C (30 s), followed by 40 cycles of 95 °C (5 s) and 60 °C (30 s). A melt curve analysis (60 °C to 95 °C at 0.15 °C/s) followed each run. The sequence analysis of the qPCR products was performed by Macrogen (Republic of Korea). For comparative analysis, the WOAH probe-based RT-qPCR was conducted using TaqMan™ Universal PCR Master Mix (Applied Biosystems, USA) with 900 nM primers and 250 nM probe (Table 2) in a 25 μL volume (WOAH, 2021; Chapter 2.3.5.). The detection performance of the designed primers was evaluated using various IHNV genotypes including U, M, JN and JS viruses as templates. Performance on E genotype was tested on infected fish samples.

2.6. Limit of detection and PCR efficiency

The limit of detection (LoD) was evaluated and compared between the SYBR Green-based qPCR assay and the WOAH probe-based RT-qPCR to assess preliminary analytical sensitivity and amplification efficiency. Ten-fold serial dilutions of pDNA-S and pDNA-W, ranging from 1.0×10^7 to 1.0×10^0 copies/reaction, were prepared.

The amplification efficiency (E) of the SYBR green-based qPCR assay and the WOAH probe-based RT-qPCR were calculated using the slope of the quantification cycle (Cq) values, expressed as $E = (10^{-1/(\text{slope})} - 1) \times 100$. Linearity was assessed by determining the coefficient of determination (R^2). Additionally, to evaluate assay robustness, the coefficient of variation (% CV) and standard deviation (SD) were calculated based on the Cq values from experimental replicates.

Table 2
List of primers and probes used in this study.

Primers and probe	Sequence (5′ → 3′)	GC	Tm	ΔG	Product size	Reference
IHNV qN 1F	GAG GAT GCA GAG ACG GAG TA	55.0	57.1	0.00	173 bp	This study
IHNV qN 2R	ATG GGA CCG TCT CTG CAA TG	55.0	58.8	−1.30		
IHNV qN 2F	ATG ACA AGC GCA CTC AGA GA	50.0	57.3	0.00	1019 bp	This study
IHNV N Partial 1R	GAC AAG GAG ACG AGC GAT CT	55.0	57.7	0.00		
IHNv N 796F	AGA GCC AAG GCA CTG TGC G	63.2	62.8	−1.6	106 bp	WOAH, 2021
IHNv N 875R	TTC TTT GCG GCT TGG TTG A	47.4	56	−0.3		
IHNV N 818 T	5′-FAM-TGA GAC TGA GCG GGA CA-MGBNFQ-3	58.8	57	0.00		

2.7. Analytical sensitivity

The analytical sensitivity (ASe) of the SYBR Green-based RT-PCR assay was evaluated as $LoD_{95\%}$ using 2-fold serial dilutions from 62.5 to 0.98 copies/reaction in 24 replicates. The starting dilution of 62.5 copies/reaction was decided from LoD, where defined as the lowest dilution yielding 100% positive detection in two independent experiments. Data analysis was conducted based on amplification curves and single melting curve plots to classify results as positive or negative. Confidence intervals (CI) for each experiment were calculated using MedCalc Statistical Software (version 20.109; MedCalc Software Ltd., Ostend, Belgium), and the $LoD_{95\%}$ CI values were derived accordingly.

2.8. Analytical specificity

To confirm the analytical specificity (ASp) of the new RT-qPCR assay, SYBR Green-based qPCR assay was performed using negative samples as templates, including uninfected rainbow trout and Atlantic salmon tissues including spleen, head kidney, gill, brain, and heart, seven cell lines (fathead minnow cell line; FHM, Atlantic salmon kidney cell line; ASK, the transgenic cell line; RTG-P1, Atlantic salmon Head Kidney cell line-1; SHK-1, *Epithelioma papulosum cyprini* cell line; EPC, chinook salmon embryo cell line; CHSE-214, rainbow trout gonad cell line-2; RTG-2), five bacteria (*Streptococcus iniae*, *Aeromonas salmonicida salmonicida*, *Vibrio anguillarum*, *Edwardsiella tarda* and *Chryseobacterium* sp.), and four viruses (Red seabream iridovirus; RSIV, Infectious salmon anaemia virus; ISAV, and Salmonid alphavirus; SAV, Viral Hemorrhagic Septicemia Virus;VHSV).

2.9. Diagnostic sensitivity and specificity

Experimental infections were performed using three IHNV genotypes to obtain positive tissue samples. In Republic of Korea, infection experiments were conducted using the JN (RtPc22) and USM (220:90) strains. Rainbow trout (average body weight 3.1 ± 0.4 g) were acclimated for two weeks at 11 ± 0.5 °C and intraperitoneally injected with 100 μL of viral inoculum containing 1.0×10^4 copies/fish.

Infection experiments with the IHNV E genotype were conducted outside Republic of Korea, as this genotype has not been reported domestically. These experiments were performed at the EU IERP facility of INRAE, France. Rainbow trout (average body weight 14.7 ± 4.0 g) were reared in a flow-through freshwater system maintained at 11 to 12 °C and intramuscularly injected with 100 μL of viral inoculum containing IHNV E (32–87) at 1.0×10^4 copies/fish.

Fish in the JN group were euthanized at 6 days post-infection (dpi), those in the M group at 9 dpi, and those in the E genotype group at 3, 6, and 8 dpi. Brain, heart, spleen, and head kidney tissues were collected according to WOAH recommendations (Ko et al., 2026).

All experimental procedures conducted in the Republic of Korea complied with the Animal Care and Use Guidelines for Animal Welfare of Gangneung-Wonju National University (GWNU-2023-29).

All animal infection experiments and sampling procedures were performed at the INRAE-IERP fish facilities authorized for animal experimentation under French regulation C78–720. The experiments

were conducted in accordance with European guidelines for the protection of animals used for scientific purposes (Directive 2010/63/EU), and the experimental protocols were reviewed and approved by the ethical committee COMETHEA (no. 45) and authorized by the French Ministry of Higher Education and Research (APAFIS no. 49598-2024051722105562).

The diagnostic performance of the SYBR Green-based qPCR assay was assessed through comparison with the WOAH probe-based RT-qPCR. For domestic infection samples, 29 JN-type and 19 M-type positive samples were analyzed. The analysis of samples from France included 56 E-type positive samples and 30 negative samples. Diagnostic sensitivity (DSe) and diagnostic specificity (DSp) were calculated in accordance with WOAH guidelines (WOAH, 2019; Chapter 1.1.2) using the following formulas: DSe = TP / (TP + FN) and DSp = TN / (TN + FP).

2.10. Repeatability

The repeatability of the SYBR Green-based qPCR assays was evaluated using three concentrations of pDNA-S (5.0×10^8, 10^6, 10^4 copies/reaction) and three tissue samples confirmed as true-positive and true-negative by the WOAH probe-based RT-qPCR. A total of nine samples were analyzed in triplicate over five consecutive testing days at two-day intervals. To evaluate operator-associated repeatability, the same samples and reagents were independently analyzed by two operators using different real-time PCR platforms (QuantStudio™ 3 Real-Time PCR System and Bio-Rad CFX96 Real-Time PCR Detection System). Standard deviation (SD) and coefficient of variation (CV) were calculated to assess assay repeatability.

2.11. Reproducibility

To evaluate the reproducibility of the SYBR Green-based qPCR assay, a multi-laboratory validation was conducted across five external laboratories, comprising 2 domestic and 4 international facilities. A total of 10 samples including 4 positive samples of IHNV (genotypes U, M, JN and JS) and 6 negative samples of L-15 medium, ISAV, and SAV (genotype 1, 2, 5 and 6) in duplicate were provided as RNA samples spotted on FTA cards (Qiagen) or provided as cDNA. One hundred μL of IHNV solution (1.0×10^6 copies/mL) was spotted onto FTA cards and air-dried for at least 3 h at room temperature. FTA cards were chosen due to their ability to lyse cells, denature proteins, and preserve nucleic acids at room temperature, thus allowing secure and convenient transport of viral RNA samples. Since international shipping of FTA cards was not always feasible, cDNA was sent to some labs. Despite the experimental PCR protocol was standardized and shared with each laboratory, different reagents were used by the testing laboratories. Detailed information on the qPCR platforms and commercial master mixes used by each participating laboratory is provided in Supplementary Table 4.

2.12. Field sample analysis

To evaluate the applicability of the developed diagnostic assay under field conditions, both domestic and international field samples were analyzed. A total of 67 domestic field samples were collected from rainbow trout farms in Republic of Korea. In addition, 56 international field samples were provided by the National Veterinary Research Institute (NVRI, Poland). All field samples were analyzed using the diagnostic assay developed in this study and were simultaneously tested using the WOAH probe-based RT-qPCR for comparative analysis. False positive samples according to the WOAH method were sequenced to confirm the virus.

Field samples collected from the Republic of Korea and Poland were anonymized using internal laboratory sample codes. The coded data did not include information that could identify sample owners or exact sampling locations. The Polish field sample panel included VHSV- and IPNV-positive samples, which were used to assess potential cross-reactivity of the IHNV SYBR Green-based qPCR assay.

3. Results

3.1. Genotype inclusivity and primer specificity

The newly developed SYBR Green-based qPCR assay successfully amplified all five major IHNV genotypes (U, M, JS, JN, and E), yielding consistent amplification plots (Fig. 1) and single melting peaks. Cq values were 13.33 (U), 14.81 (M), 14.83 (JN), 16.69 (JS), and 16.38 (E) in SYBR Green-based qPCR assay while 14.36 (U), 16.12 (M), 16.42 (JN), 20.30 (JS), 18.69 (E) in WOAH probe-based RT-qPCR, with no amplification detected in the negative controls (Table 3). Indeed, the WOAH probe-based RT-qPCR exhibited suboptimal performance for the JS genotype (RTYK1801), characterized by atypical amplification curves and weak fluorescence signals (Fig. 1). Sequence analysis showed that the reduced efficiency was caused by a single nucleotide polymorphism in the probe-binding region, with a C-to-T substitution at the 11th nucleotide of the probe-binding sequence (Figs. 2 and 3).

3.2. Limit of detection and PCR efficiency

LoD and PCR efficiency were assessed using tenfold serial dilutions of pDNAs ranging from 5.0×10^7 to 5.0×10^0 copies/reaction. LoD of SYBR Green-based qPCR using pDNA-S was 5.0×10^0 copies/reaction, with a corresponding Cq value of 35.34 (Table 4). The assay demonstrated a linear correlation with a coefficient of determination (R^2) of 0.991, a slope of −3.54, and an amplification efficiency of 91.48%. The WOAH method using pDNA-W yielded an LoD of 5.0×10^3 copies/reaction with a Cq value of 34.95 (Table 4), exhibiting a linear correlation with $R^2 = 0.999$, a slope of −3.5, and an amplification efficiency of 93.07%.

3.3. Analytical sensitivity

The $LoD_{95\%}$ of the SYBR Green-based qPCR assay was determined by performing twofold serial dilutions of pDNA-S (starting from 62.5 copies/reaction) in 24 replicates. The detection limit was initially defined based on the lowest concentration that achieved 100% detection across three replicates. The estimated $LoD_{95\%}$ for IHNV was 16.92 copies/reaction, with a 95% confidence interval (CI) of 14.08–21.82 copies/reaction (Fig. 4).

3.4. Analytical specificity

The SYBR Green-based qPCR assay produced amplification signals exclusively in IHNV-positive samples. No Cq values were detected in uninfected fish tissues (rainbow trout and Atlantic salmon), fish cell lines (FHM, ASK, RTG-P1, SHK-1, EPC, CHSE-214, and RTG-2), bacterial pathogens (*Streptococcus iniae*, *Aeromonas salmonicida salmonicida*, *Vibrio anguillarum*, *Edwardsiella tarda*, and *Chryseobacterium* sp.), or other fish viruses (RSIV, ISAV, SAV and VHSV) (Table 5). Consistent with this result, VHSV- and IPNV-positive samples included in the Polish field sample panel did not show amplification in the IHNV SYBR Green-based qPCR assay (Supplementary Table 10). Melting curve analysis further confirmed the absence of nonspecific amplification, indicating high analytical specificity of the assay (data not shown).

3.5. Diagnostic sensitivity and specificity

To evaluate the diagnostic performance of the SYBR Green-based qPCR assay, a total of 134 samples were analyzed, including 29 JN-type, 19 M-type, and 56 E-type positive samples, as well as 30 negative samples, as determined by the WOAH probe-based RT-qPCR (Supplementary Table 5, 6, and 7). The SYBR Green-based qPCR assay showed complete agreement with the reference method, correctly

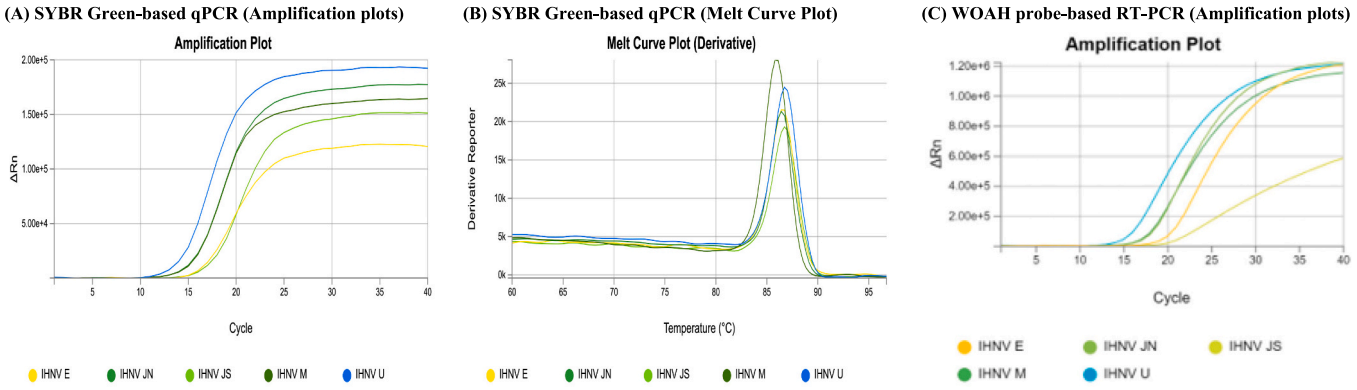

Fig. 1. Amplification plots and genotype detection results of IHNV using SYBR Green-based qPCR (A and B) and WOAH probe-based RT-PCR (C) assays. Amplification plots generated for detecting various IHNV genotypes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Comparison of quantification cycle (Cq) values obtained from SYBR Green-based and WOAH probe-based RT-PCR assays for five IHNV genotypes.

Sample	SYBR Green-based RT-PCR		WOAH probe-based RT-PCR	
	Cq Mean	Cq SD	Cq Mean	Cq SD
IHNV U	13.33	0.14	14.36	0.01
IHNV M	14.81	0.03	16.12	0.36
IHNV JN	14.83	0.05	16.42	0.28
IHNV JS	16.69	0.06	20.30	0.13
IHNV E	16.38	0.07	18.69	0.16

identifying all positive and negative samples (Table 6). The assay demonstrated 100% DSe and 100% DSp, with no false positives or false negatives observed.

3.6. Repeatability

The CV for IHNV across different experimenters ranged from 0.02% to 1.37% (Table 7), and across different days ranged from 0.59% to 3.83% (Table 8). The correlation analysis conducted between the two experimenters yielded an R^2 value of 0.9388 (Fig. 5). These results confirm that the SYBR Green-based qPCR assay for IHNV exhibits high repeatability and consistent performance across operators and testing dates.

3.7. Reproducibility

The inter-laboratory reproducibility of the newly developed SYBR Green-based qPCR assay was rigorously evaluated through a multi-laboratory ring test involving seven independent facilities (Laboratories A–G). To reflect real-world diagnostic conditions, while the experimental protocol was standardized, the participating laboratories utilized their own diverse reagents and different operators and PCR platforms to conduct the analysis. All laboratories accurately identified the four IHNV-positive samples, yielding consistent results. Furthermore, no false-positive or false-negative results were reported for any of the negative controls or non-target viral pathogens (Table 9).

Fig. 2. Nucleic acid sequence analysis of IHNV primers designed in this study. Bold text highlights the primer regions, and red text indicates mismatches between the primer and the base sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

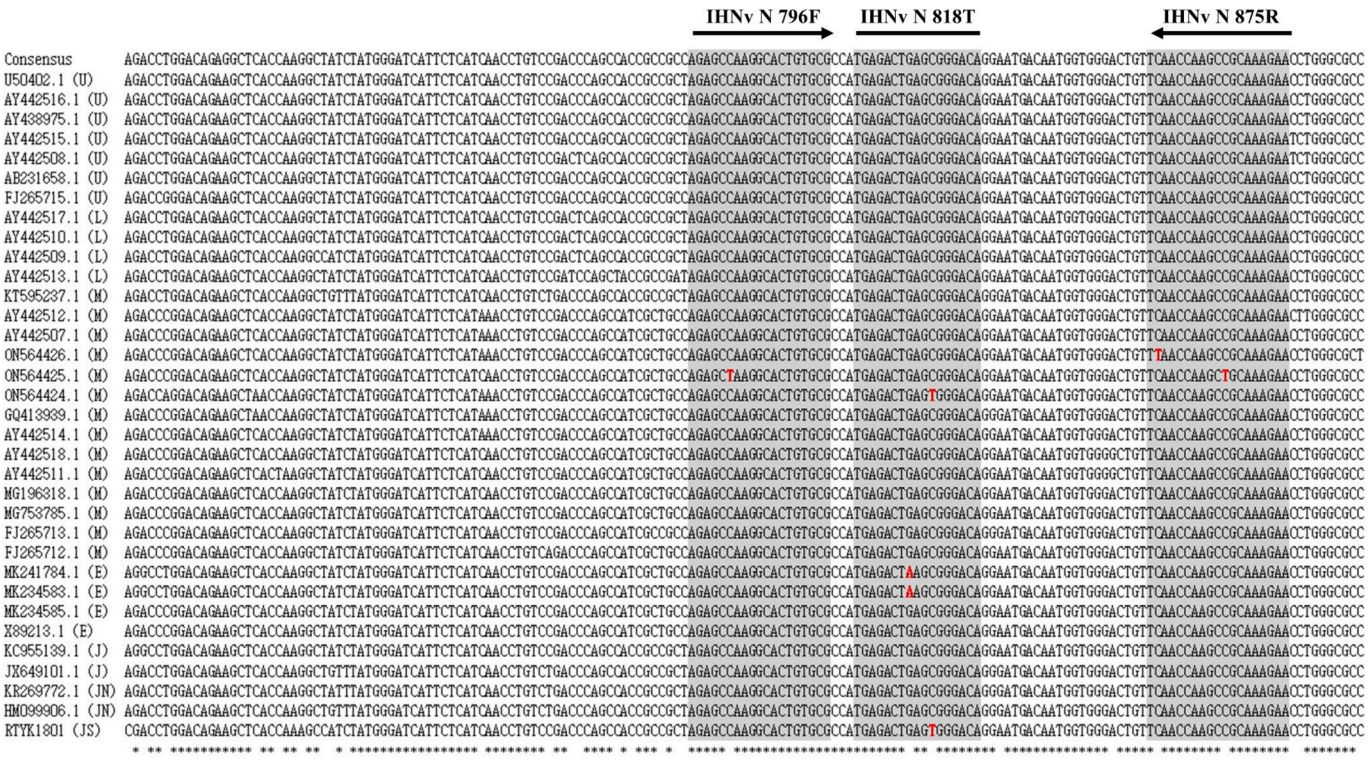

Fig. 3. Location of primers and probes for IHNV recommended by WOAH. Bold text highlights the primer regions, and red text indicates mismatches between the primers and the base sequences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Comparison of standard curve parameters and analytical sensitivity between the developed SYBR Green-based qPCR assay and the WOAH-recommended probe-based RT-PCR assay for IHNV detection.

	SYBR Green-based qPCR	WOAH probe-based RT-PCR
Slope	−3.545	−3.500
Efficiency (%)	91.475	93.073
Y-intercept	36.833	45.479
R^2	0.964	0.999
Standard error	0.058	0.040
Limit of detection (Cq)	35.34	34.97

3.8. Application of field samples

For the domestic field samples, the initial diagnostic performance analysis was performed using the WOAH probe-based RT-qPCR assay as the reference comparator. In this WOAH-based initial analysis, 23 positive and 44 negative samples were identified using the WOAH probe-based RT-qPCR assay, whereas 30 positive and 37 negative samples were detected using the SYBR Green-based qPCR assay (Supplementary Table 8). A total of 11 discordant results were observed, including nine WOAH-negative/SYBR-positive samples and two WOAH-positive/SYBR-negative samples. Under this initial interpretation, the DSe and DSp of the SYBR Green-based qPCR assay were 91.3% (21/23) and 79.5% (35/44), respectively (Table 10A).

Among the nine WOAH-negative/SYBR-positive discordant samples, eight were confirmed as IHNV-positive by sequencing of the qPCR

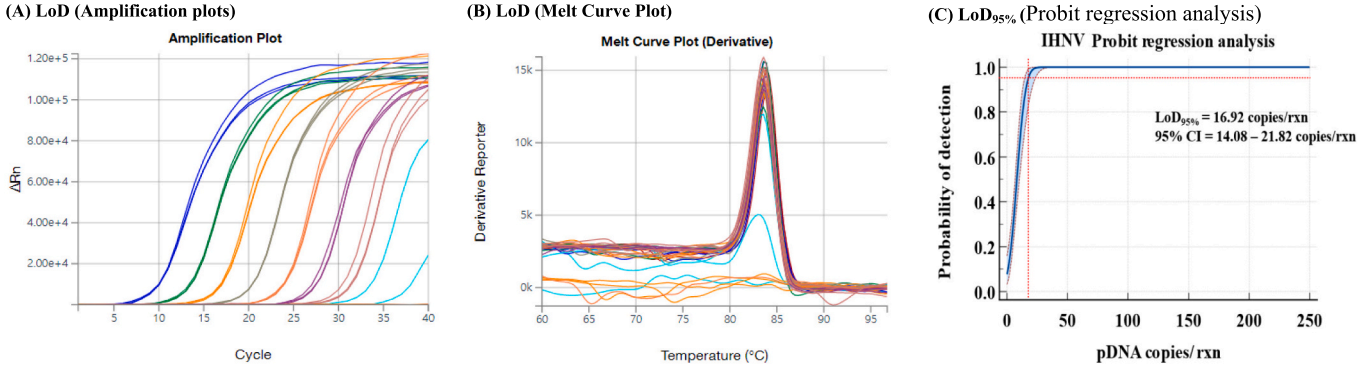

Fig. 4. Determination of the $LoD_{95\%}$ of the IHNV SYBR Green-based qPCR assay. (A) Amplification plots generated from serially diluted pDNA standards. (B) Melt curve analysis showing a single specific melting peak for the amplified IHNV target. (C) Probit regression analysis used to estimate the 95% limit of detection ($LoD_{95\%}$). The x-axis represents the pDNA copy number per reaction, and the y-axis represents the probability of detection. The LoD95% was estimated to be 16.92 copies/reaction, with a 95% confidence interval of 14.08–21.82 copies/reaction. The red dotted line indicates the 95% detection probability threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Analytical specificity of SYBR Green-based qPCR assay and WOAH probe-based RT-PCR assay.

No.	Samples	SYBR Green-based qPCR	WOAH probe-based RT-PCR
1	*S. salar* spleen	–	–
2	*S. salar* head kidney	–	–
3	*S. salar* gill	–	–
4	*S. salar* brain	–	–
5	*S. salar* heart	–	–
6	*O. mykiss* spleen	–	–
7	*O. mykiss* head kidney	–	–
8	*O. mykiss* gill	–	–
9	*O. mykiss* brain	–	–
10	*O. mykiss* heart	–	–
11	FHM	–	–
12	ASK	–	–
13	RTG P1	–	–
14	SHK-1	–	–
15	EPC	–	–
16	CHSE-214	–	–
17	RTG-2	–	–
18	*A. salmonicida salmonicida*	–	–
19	*V. anguillarum*	–	–
20	*E. tarda*	–	–
21	*S. iniae*	–	–
22	*Chryseobacterium* sp.	–	–
23	ISAV	–	–
24	RSIV	–	–
25	SAV	–	–
26	VHSV	–	–

+: amplification, −: non-amplification.

Table 6
Diagnostic performance of the SYBR Green-based qPCR assay using experimentally infected samples with known infection status.

SYBR Green-based qPCR		WOAH probe-based RT-qPCR	
		Known Positive (104)	Known Negative (30)
Test result	Positive	104 (TP)	0 (FP)
	Negative	0 (FN)	30 (TN)
DSe = TP / (TP + FN)		100% (104 / 104)	
DSp = TN / (TN + FP)		100% (30 / 30)	

Table 7
Between-Day repeatability verification of IHNV SYBR Green-based qPCR assay.

Between-Day		10^8	10^6	10^4	IHNV M	IHNV JN	
1 day	Cq Mean	8.66	15.84	22.83	19.14	19.25	20.13
	Cq SD	0.02	0.02	0.11	0.03	0.05	0.03
	CV (%)	0.22	0.11	0.48	0.16	0.25	0.14
3 days	Cq Mean	8.95	15.85	22.83	19.5	19.64	19.90
	Cq SD	0.07	0.22	0.01	0.04	0.11	0.12
	CV (%)	0.76	1.37	0.05	0.21	0.55	0.59
5 days	Cq Mean	8.99	15.99	23.01	19.43	19.45	20.21
	Cq SD	0.02	0.07	0.11	0.00	0.11	0.05
	CV (%)	0.22	0.43	0.46	0.02	0.58	0.27
7 days	Cq Mean	9.40	16.35	23.1	20.00	20.83	20.10
	Cq SD	0.12	0.08	0.15	0.07	0.03	0.04
	CV (%)	1.31	0.48	0.65	0.35	0.14	0.21
9 days	Cq Mean	8.51	15.42	22.07	19.37	19.41	20.02
	Cq SD	0.04	0.05	0.19	0.03	0.08	0.04
	CV (%)	0.45	0.35	0.86	0.18	0.43	0.19

products (Supplementary Table 9, Fig. 4 and 5). In the sequencing-resolved interpretation, these eight samples were reclassified as true positives, resulting in recalculated DSe and DSp values of 93.5% (29/31) and 97.2% (35/36), respectively (Table 10B).

For international samples, analysis was conducted using 37 positive and 19 negative samples confirmed by the WOAH probe-based RT-qPCR. The SYBR Green-based qPCR assay detected 34 positive and 22 negative samples (Supplementary Table 6). The diagnostic results for IHNV showed 34 true positives (TP), 0 false positives (FP), 3 false negatives (FN), and 19 true negatives (TN). Accordingly, the DSe was 91.8% (34/37), and the DSp was 100% (19/19) (Table 11).

The field validation results are summarized in Table 12. In the domestic cohort, 67 samples were tested, and 11 discordant results were observed between the WOAH and SYBR assays. Among the nine WOAH-negative/SYBR-positive samples, eight were confirmed as IHNV-positive by sequencing. In the Polish cohort, 56 samples were tested, and three discordant results were observed, with no WOAH-negative/SYBR-positive samples detected.

4. Discussion

This study addresses a critical limitation of the WOAH probe-based RT-qPCR assay when sequence variation occurs within the probe-binding region. Among the tested IHNV genotypes, the largest Cq difference between the two assays was observed for the JS genotype. The WOAH probe-based RT-qPCR assay showed an approximately 3.6-Cq delay compared with the SYBR Green-based qPCR assay for this genotype. Because both assays used the same cDNA preparation and equivalent template input, this difference was unlikely to be caused by sample loading variation. Instead, MSA analysis showed that the JS genotype sequence RTYK1801 contains nucleotide mismatches within the WOAH N-gene probe-binding region. These mismatches may reduce probe hybridization efficiency and contribute to delayed fluorescence signal accumulation in the probe-based assay. In contrast, the primer-binding regions of the newly developed SYBR Green-based assay were conserved across the tested IHNV genotypes, supporting its stable detection of the JS lineage.

Our SYBR Green-based qPCR assay targets the conserved region of the IHNV N gene. The N gene is known for its genetic stability and high transcription level, making it a suitable target for molecular diagnostics. Targeting conserved genomic regions enables reliable primer binding and facilitates the detection of diverse IHNV genotypes while maintaining high analytical sensitivity and specificity (Chico et al., 2006; Purcell et al., 2013). The designed primer set successfully detected multiple IHNV genotypes, including U, M, E, JN, and JS, demonstrating broad genotype coverage for IHNV detection. While the selection of conserved regions within the N gene provides the basis for broad genotype inclusivity, the N gene's genetic stability and high transcription level make it an ideal target for molecular diagnostics (Kurath and Leong, 1985; Chico et al., 2006; Purcell et al., 2013).

The careful adjustment of primer GC content, *Tm*, and ΔG was important for minimizing non-specific amplification and primer-dimer formation, which are common limitations of SYBR Green-based qPCR assays (Bustin et al., 2005; Wong and Medrano, 2005). Moreover, the newly designed primers were located in conserved regions of the IHNV N gene, enabling stable amplification across the tested genotypes, including the JS genotype. In contrast, the WOAH probe-based RT-qPCR assay showed delayed detection of the JS genotype, which was likely associated with a single nucleotide mismatch within the probe-binding region and reduced probe hybridization efficiency (Hoferer et al., 2019; Purcell et al., 2013). This interpretation is consistent with the Cq difference observed for the JS genotype, in which the SYBR Green-based qPCR assay yielded a Cq value of 16.69, whereas the WOAH probe-based RT-qPCR assay yielded a delayed Cq value of 20.30.

$LoD_{95\%}$ is a critical parameter in molecular diagnostic validation because it represents the target concentration that can be detected with 95% probability. Unlike a conventional LoD, which may indicate the lowest concentration detected under a specific experimental condition, $LoD_{95\%}$ provides a more robust estimate of analytical sensitivity and assay reliability (Bustin et al., 2009; Forootan et al., 2017). In this study, the IHNV SYBR Green-based qPCR assay showed a $LoD_{95\%}$ of 16.92

Table 8
Between-technician repeatability verification of the IHNV SYBR Green-based qPCR assay.

	10^8		10^6		10^4		IHNV M		IHNV J		Tissue	
Technician	A	B	A	B	A	B	A	B	A	B	A	B
Mean (Cq)	8.9	9.68	15.89	16.64	22.77	23.45	19.49	18.94	19.72	19.03	N/A	N/A
SD (Cq)	0.34	1.22	0.33	0.93	0.41	1.00	0.32	0.42	0.64	0.48	0	0
CV (%)	3.83	12.59	2.11	5.61	1.79	4.27	1.63	2.22	3.25	2.54	0	0

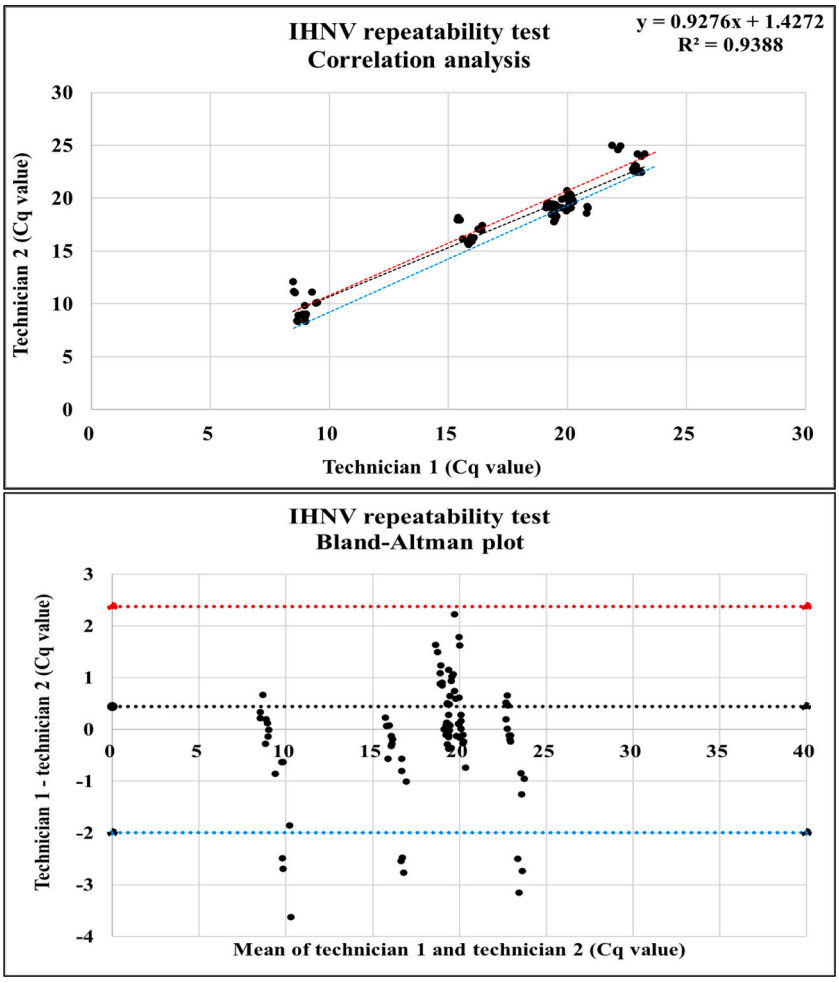

Fig. 5. Repeatability verification of the IHNV SYBR Green-based qPCR assay. Correlation and Bland-Altman analysis of Cq values for technicians 1 and 2 for IHNV pDNA (1.0×10^8, 10^6, 10^4), IHNV M, and IHNV JN. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

copies/reaction (95% CI: 14.08–21.82 copies/reaction), indicating reproducible detection of low-copy IHNV templates.

Several IHNV-specific real-time PCR assays have previously reported analytical sensitivity as conventional LoD or detection limit values rather than $LoD_{95\%}$. Dhar et al. (2008) developed an IHNV-specific SYBR Green real-time RT-PCR assay targeting the N and G genes for detection and quantification of IHNV in rainbow trout; however, $LoD_{95\%}$-based analytical sensitivity was not clearly established in that study. Purcell et al. (2013) reported a universal N gene-based TaqMan RT-qPCR assay for IHNV detection and validation across representative IHNV strains. Batts et al. (2022) reported a U/M genogroup-discriminating multiplex RT-rPCR assay with LoD values of <10 copies/reaction for artificial positive control constructs and < 30 copies/reaction for viral RNA. More recently, Zheng et al. (2025) reported a DPO-based real-time RT-PCR assay with a minimum detection limit of 36.2 copies/μL.

Although direct numerical comparison between LoD and $LoD_{95\%}$ should be interpreted cautiously because the template type, reaction format, unit, and calculation method differ among studies, the $LoD_{95\%}$ of 16.92 copies/reaction obtained in the present study indicates that the newly developed SYBR Green-based qPCR assay achieved analytical sensitivity comparable to established IHNV real-time PCR assays. Importantly, unlike previous IHNV SYBR Green-based assays in which $LoD_{95\%}$-based sensitivity was not clearly defined, the present study provides a probability-based analytical sensitivity estimate together with conserved N gene-based primer optimization, broad genotype detection, direct comparison with the WOAH probe-based RT-qPCR assay, field sample validation, and multi-laboratory applicability.

Repeatability testing confirmed the reliability of the newly developed assay under repeated analytical conditions. Using identical standard samples and reagents, the CV values ranged from 0.02% to 1.37% among operators and from 0.59% to 3.83% across different testing dates, remaining below the commonly accepted 5% threshold for qPCR-

Table 9
Reproducibility test results for IHNV detection.

No.	Samples	Laboratories						
		A	B	C	D	E	F	G
1	L-15 medium	–	–	–	–	–	–	–
2	L-15 medium	–	–	–	–	–	–	–
3	IHNV U	+	+	+	+	+	+	+
4	IHNV U	+	+	+	+	+	+	+
5	IHNV M	+	+	+	+	+	+	+
6	IHNV M	+	+	+	+	+	+	+
7	IHNV JN	+	+	+	+	+	+	+
8	IHNV JN	+	+	+	+	+	+	+
9	IHNV JS	+	+	+	+	+	+	+
10	IHNV JS	+	+	+	+	+	+	+
11	SAV1	–	–	–	–	–	–	–
12	SAV1	–	–	–	–	–	–	–
13	SAV2	–	–	–	–	–	–	–
14	SAV2	–	–	–	–	–	–	–
15	SAV5	–	–	–	–	–	–	–
16	SAV5	–	–	–	–	–	–	–
17	SAV6	–	–	–	–	–	–	–
18	SAV6	–	–	–	–	–	–	–
19	ISAV	–	–	–	–	–	–	–
20	ISAV	–	–	–	–	–	–	–

Table 10
Diagnostic performance of the SYBR Green-based qPCR assay in domestic field samples (Republic of Korea).

(A). Analysis using the WOAH probe-based RT-qPCR assay as the reference comparator			
		WOAH probe-based RT-qPCR	
SYBR Green-based qPCR		Known Positive (23)	Known Negative (44)
Test result	Positive	21 (TP)	9 (FP)
	Negative	2 (FN)	35 (TN)
DSe = TP / (TP + FN)		91.3% (21 / 23)	
DSp = TN / (TN + FP)		79.5% (35 / 44)	
(B) Sequencing-resolved interpretation of discordant samples			
		SYBR Green-based qPCR+Sequencing	
SYBR Green-based qPCR		Known Positive (31)	Known Negative (36)
Test result	Positive	29 (TP)	1 (FP)
	Negative	2 (FN)	35 (TN)
DSe = TP / (TP + FN)		93.5% (29 / 31)	
DSp = TN / (TN + FP)		97.2% (35 / 36)	

Table 11
Diagnostic performance of the SYBR Green-based qPCR assay in International field samples (Poland).

SYBR Green-based qPCR		NVRI*	
		Known Positive (37)	Known Negative (19)
Test result	Positive	34 (TP)	0 (FP)
	Negative	3 (FN)	19 (TN)
DSe = TP / (TP + FN)		91.8% (34 / 37)	
DSp = TN / (TN + FP)		100% (19 / 19)	

*NVRI (National Veterinary Research Institute, Poland): Real time RT-PCR, ELISA, G gene sequencing (WOAH)

based assays (Jennings et al., 2009; Food and Drug Administration (FDA), 2018). Although some dispersion in Cq values was observed, this may be partly attributed to the use of different qPCR instruments by different operators, as fluorescence detection systems, thermal cycling performance, baseline correction, and threshold-setting algorithms can vary between platforms. Nevertheless, the assay consistently detected the target and maintained acceptable CV values, indicating that the observed dispersion did not substantially affect assay repeatability.

Reproducibility was further assessed through inter-laboratory testing. The robustness of a molecular diagnostic tool is best demonstrated by its consistent performance under diverse laboratory conditions (Bustin et al., 2009; Burd, 2010). In this study, the inter-laboratory reproducibility test yielded 100% agreement among seven different facilities, including two domestic and five international institutions. A key strength of these findings is that the assay remained highly reliable even when laboratories employed different commercial reagents and various PCR platforms. This high level of reproducibility is particularly significant for SYBR Green-based methodologies, as it confirms that the meticulous thermodynamic optimization of our primer set successfully eliminated the risk of reagent-specific non-specific signals or primer-dimer formation (Thornton and Basu, 2011; Kubista et al., 2006). The ability of the assay to reliably distinguish IHNV from other common salmonid pathogens across multiple facilities, despite variations in equipment and laboratory-specific resources, underscores its potential as a standardized diagnostic tool. Consequently, these results support the global applicability of this assay for large-scale national surveillance and routine monitoring programs.

In the field sample analysis, discordant results were observed between the WOAH probe-based RT-qPCR assay and the newly developed SYBR Green-based qPCR assay. In the domestic cohort, nine samples that were negative by the WOAH assay were positive by the SYBR Green-based assay. To determine whether these results represented true false positives, sequencing analysis was performed, and IHNV-specific sequences were confirmed in eight of the nine discordant samples. This result indicates that most of the WOAH-negative/SYBR-positive samples were not caused by non-specific amplification of the SYBR Green-based assay, but represented sequencing-confirmed IHNV-positive discordant samples.

In the domestic field sample analysis, the estimated diagnostic performance varied depending on the interpretation criteria. When the WOAH assay was used as the reference comparator, the DSe and DSp were 91.3% and 79.5%, respectively. After sequencing-resolved reclassification of eight WOAH-negative/SYBR-positive samples, the recalculated DSe and DSp increased to 93.5% and 97.2%, respectively. This highlights that diagnostic performance in field samples can be influenced by the reference method and how discordant results are interpreted.

In contrast, no WOAH-negative/SYBR-positive results were observed in the Polish cohort, indicating that no false-positive amplification was detected in this cohort under the tested conditions. The different discordance pattern between the domestic and Polish cohorts may be associated with regional differences in IHNV genotypes or sequence variation within the WOAH target region. Because IHNV strains reported in the Republic of Korea mainly belong to the J genotype, nucleotide variation in the WOAH primer/probe-binding region may have contributed to reduced detection efficiency of the probe-based assay in some domestic field samples. Also, these discordant results may have been influenced by low viral RNA levels near the detection

Table 12
Summary of field sample validation and discordant result analysis.

Cohort	No. of samples	WOAH positive	WOAH negative	SYBR positive	SYBR negative	WOAH-negative / SYBR-positive	WOAH-positive / SYBR-negative	Sequencing confirmation
Republic of Korea	67	23	44	30	37	9	2	8/9 WOAH-negative/SYBR-positive samples were sequencing-confirmed as IHNV
Poland	56	37	19	34	22	0	3	No false-positive amplification was observed

limit, differences in RNA quality or sample preservation, extraction efficiency, or possible sequence variation affecting primer-binding efficiency.

Despite its advantages, the SYBR Green-based assay has inherent limitations. Because SYBR Green detects any double-stranded DNA, non-specific amplification and primer-dimer formation can produce fluorescence signals. Therefore, positive results should be interpreted together with melting curve analysis, expected Tm values, and, when necessary, sequencing confirmation (Bustin et al., 2009; Forootan et al., 2017). When applied with these interpretive criteria, the field sample data indicate that the SYBR Green-based qPCR assay can serve as a practical screening tool for IHNV detection in diagnostic laboratories and field applications.

Overall, these findings demonstrate that the SYBR Green-based qPCR assay developed in this study provides a sensitive, reproducible, and cost-effective approach for IHNV detection. Its broad genotype coverage, low $LoD_{95\%}$, high repeatability, and inter-laboratory reproducibility support its potential application in routine surveillance, disease monitoring, and quarantine programs in aquaculture systems. When combined with melting curve analysis and confirmatory testing for discordant samples, this assay may serve as a practical complementary tool to existing probe-based diagnostic methods.

CRediT authorship contribution statement

Sungjae Ko: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Formal analysis, Data curation, Conceptualization. **Eunsu Song:** Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Data curation. **Magdalena Stachnik:** Validation, Software, Methodology, Formal analysis, Data curation. **Bertrand Collet:** Writing – review & editing, Validation, Formal analysis, Data curation. **Bartolomeo Gorgoglione:** Writing – review & editing, Validation, Methodology, Data curation. **Po-Tsang Lee:** Validation, Formal analysis, Data curation. **Alejandro J. Yáñez:** Validation, Formal analysis, Data curation. **Joon Bum Jeong:** Validation, Formal analysis, Data curation. **Kwangil Kim:** Validation, Formal analysis, Data curation. **Youngchul Kim:** Validation, Methodology, Formal analysis, Data curation. **Dimitri Rigaudeau:** Validation, Methodology. **Mun Gyeong Kwon:** Supervision, Resources, Project administration, Funding acquisition. **Jae-Ok Kim:** Supervision, Resources, Project administration, Funding acquisition. **Min Jae Kim:** Supervision, Resources, Project administration, Funding acquisition. **Ji-Min Jeong:** Supervision, Resources, Project administration, Funding acquisition. **Suhee Hong:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Consent for publication

Not applicable.

Ethics approval

All experimental procedures conducted in the Republic of Korea complied with the Animal Care and Use Guidelines for Animal Welfare of Gangneung-Wonju National University (GWNU-2023-29).

All animal infection experiments and sampling procedures were performed at the INRAE-IERP fish facilities authorized for animal experimentation under French regulation C78-720. The experiments were conducted in accordance with European guidelines for the protection of animals used for scientific purposes (Directive 2010/63/EU), and the experimental protocols were reviewed and approved by the ethical committee COMETHEA (no. 45) and authorized by the French Ministry of Higher Education and Research (APAFIS no. 49598-2024051722105562).

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2026.744469.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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