

## Preparation and optimization of rapid and sensitive coagglutination test for detection of infectious pancreatic necrosis virus (IPNV)

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**Abstract:** The aim of this study is the development of a coagglutination test for rapid diagnosis of infectious pancreatic necrosis (IPN) virus which causes an acute infectious viral disease with high mortality in young salmonid fish. Serotypes Sp, Ab, WB of the IPNV were cultivated in BF-2 cells and purified by linear sucrose density gradient centrifugation. Hyperimmune sera against purified IPNV serotypes were collected from immunized guinea pigs. Coagglutination test conjugates were prepared by using sensitized *S. aureus* and hyperimmune sera. Cell culture derived salmonid pathogens such as infectious hematopoietic necrosis virus (IHNV), viral haemorrhagic septicaemia virus (VHSV) and epizootic hematopoietic necrosis virus (EHNV) were used to determine the specificity of the test as control. Upward comparison of the preferability for the rapid diagnosis was also carried out with ELISA and RT-PCR. The validation studies of newly developed coagglutination test were carried out in accordance with the Manual of Diagnostic Tests for Aquatic Animals. Consequently, determined detection limit of the test was approximately  $10^{5.25}$  TCID<sub>50</sub>/mL for the Sp and Ab serotypes and  $10^{7.75}$  TCID<sub>50</sub>/mL for the WB in laboratory conditions. The results indicated that newly developed coagglutination test was compatible with ELISA and RT-PCR in positive cell culture supernatants. It was concluded that this test would be an economic, rapid and reliable method for the diagnosis of IPN virus in cell culture.

**Key words:** Infectious pancreatic necrosis virus, coagglutination, trout, salmonid

### 1. Introduction

Infectious pancreatic necrosis (IPN) is an acute infectious viral disease that causes significant economic losses due to its high mortality, especially in young salmonid fish [1,2]. The causative agent is the infectious pancreatic necrosis virus (IPNV), which belongs to the genus Aquabirnavirus within the Birnaviridae family [3].

In serological classification, the virus has 2 serogroups, namely A and B. While serogroup A contains nine serotypes from A1 to A9 (A1: WB, A2: Sp, A3: Ab, A4: HE, A5: Te, A6: Can-1, A7: Can-2, A8: Can-2, A9: Ja), serogroup B contains only one serotype (B1: TV-1) [4]. There are 6 genogroups reported, based on the amino acid similarity of the VP2 gene. This classification is also compatible with the serological and geographic origin of the isolates [5]. A new genogroup was reported based on comparison of nucleotide sequences from the VP2/NS junction region, and it was thought to be a new serotype within serogroup A [6]. The genetic analysis shows that natural reassortment occurs among the genogroups [7].

Although the agent mainly induces disease in salmonids, it has been isolated from over 100 different species, and these species may be the source of the spread of the virus. IPNV is widespread all over the world except in Australia and Iceland [8].

IPN infection is also common in trout farms in Turkey, with high mortality rates in offspring. The virus remains in circulation for years in farms without clinical symptoms, whereas infection through carriers is identified [9].

IPNV can be detected using several methods [10–13] but generally, antibody-based tests such as enzyme-linked immunosorbent assay (ELISA), immunofluorescent antibody test (IFAT) and coagglutination test (COA) following virus isolation in cell culture are preferred by reference laboratories [8, 14, 15].

The advantages of the COA are that it can be used in the field, yields rapid results, is cost-effective, and does not require special equipment or trained personnel. The aim of this study was to develop and optimize the COA, a serological technique for rapid diagnosis of IPNV.

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## 2. Materials and methods

### 2.1. Cell culture

Bluegill fry (BF-2) and epithelioma papillosum cyprini (EPC) cell lines (obtained from the Federal Research Centre for Animal Diseases, Germany) were grown in Eagle's minimal essential medium (E-MEM) containing 10% fetal bovine serum, 1% HEPES with 100 IU/mL penicillin, 10 µg/mL streptomycin and 0.025 mg/mL amphotericin B.

### 2.2. Viruses and virus purification

IPNV (Sp), EHNV (obtained from the Danish Food and Veterinary Research Institute), IPNV (Ab), IPNV (WB), VHSV, and IHNV (obtained from the Federal Research Centre for Animal Diseases, Germany) were used as reference viruses. The TCID<sub>50</sub> titres of the propagated viruses were determined by microtitration assay [16].

The IPN virus serotypes (Sp, Ab and WB) suspensions were frozen, thawed, and centrifuged at 1500 × g for 15 min at 4 °C separately. The supernatants were collected, centrifuged at 90,000 × g for 1 h at 4 °C and the pellets were dissolved in TNE solution (0.01 M Tris, 0.1 M Sodium chloride, 1 M Ethylenediaminetetra-acetic acid). The solutions were placed on linear sucrose density gradient (15–45%) and centrifuged at 45,000 × g for 2 h at 4 °C. After centrifugation, the opaque bands containing the virus were removed and diluted in TNE, then centrifuged again at 90,000 × g for 2 hours at 4 °C. The final pellets were resuspended in TNE and incubated overnight at 4 °C. Protein concentrations were determined using the spectrophotometric method ( $A_{260} / A_{280}$ ) and suspensions were stored at –80 °C until used [9,17].

### 2.3. Production and purification of sera

Polyclonal antisera was produced according to the previously described method with some modifications [18,19]. Briefly, albino guinea pigs were immunized subcutaneously with purified virus (200 µg/ 500 µL) and 500 µL Freund's incomplete adjuvant (FIA) mixture 2 times with 3 week intervals. Blood samples were collected ten days after the last immunization, and sera were removed by centrifugation (2000 × g, 10 min). After heat inactivation (30 min, 56 °C) and purification with Protein A Antibody Purification Kit (Pure1A, Sigma), SN<sub>50</sub> titres vs. 100TCID<sub>50</sub> were determined with a microneutralisation assay [20].

### 2.4. Preparation of *S. aureus*

*S. aureus* Cowan 1 (ATCC, 12598) was cultured in blood agar for 24 h at 37 °C. The colonies were collected and transferred into brain-heart infusion broth (BHI) medium for pre-enrichment and incubated overnight at 37 °C. The harvested culture was inoculated in tryptic soy broth (TSB) and incubated at 37 °C for 24 h. The culture was centrifuged (2260 × g, 30 min). Cells were washed

with phosphate buffer saline (PBS: 0.03 M KH<sub>2</sub>PO<sub>4</sub>, 0.12 M NaCl, pH 7.3) two times and resuspended in PBS containing 0.5% formalin. The suspension was then incubated at 25 °C for 3 h followed by 80 °C for 1 h. The suspension was washed with PBS three times, and the cells were resuspended in PBS (10% v/v) containing 0.02% sodium azide [21,22].

### 2.5. Conjugate preparation

Antisera volumes of 125 mL, 250 mL and 500 mL were mixed with 1/10, 1/100, and 1/200 diluted *S. aureus* suspensions, respectively (Table 1). The solutions were incubated at 25 °C for 3 h, stirred at 30 min intervals, and finally centrifuged (2810 × g, 5 °C) for 1 h. The pellet was dissolved in PBS and stored at 4 °C until used [22].

### 2.6. Validation and detection limit of COA test

Test validation was carried out according to the Manual of Diagnostic Tests for Aquatic Animals [23]. A total of 30 positive samples, which consisted of 10 positive reference viruses, 10 positive tissue homogenates, and their cell culture supernatants, as well as 30 negative samples, which consisted of 10 negative reference viruses, 10 negative fish tissue homogenates and their cell culture supernatants were used. The formulas used in the calculation and validation test design are presented in Table 2.

For determination of detection limit, 2× diluted virus suspensions (from 1/1 to 1/1024) and controls (PBS, virus-free cell culture supernatant) were tested using the prepared conjugate. The results were evaluated in terms of the strength of the COA, generation time and whether or not the conjugate reacted with the control antigens [22].

### 2.7. Fish samples and preparation

In this study, a total of 900 trout samples were used and sample pools were created from the internal organs'

Table 1. Rates of antisera and *S. aureus* used in preparation of conjugates.

Dilution ( <i>S. aureus</i> )	Conjugate numbers	Amount of <i>S. aureus</i> (mL)	Amount of antisera (µL)
1:10	1	2	125
	2	2	250
	3	2	500
1:100	4	2	125
	5	2	250
	6	2	500
1:200	7	2	125
	8	2	250
	9	2	500
1:1	10	0.2	200

Table 2. Validation test design and calculation formula.

	Test Results		
	Known Positive	Known Negative	Total
Positive	TP (true positive)	FN (false negative)	TP + FN
Negative	FP (false positive)	TN (true negative)	FP + TN
Total	TP + FP	FN + TN	N
Sensitivity	: $[TP / (TP + FN)] \times 100 = \%$		
Specificity	: $[TN / (TN + FP)] \times 100 = \%$		
False positive rate (FPR)	: $[FP / (FP + TN)] \times 100 = \%$		
False negative rate (FNR)	: $[FN / (FN + TP)] \times 100 = \%$		

(spleen, liver, kidney and hearts) tissue sections of 10 fish. Sample preparation was carried out according to the method described in the Manual of Diagnostic Tests for Aquatic Animals [23].

### 2.8. Virus isolation in cell culture

A total of 100 mL of each fish sample homogenate were used to inoculate BF-2 cells. After seven days incubation at 15 °C, cultures were detected and examined for cytopathic effect (CPE) and toxicity. The inoculated cells were then subcultivated twice. CPE positive cultures were centrifuged (1500 × g, 15 min) and supernatants were collected and analysed comparatively with COA, ELISA, and RT-PCR.

### 2.9. Viral RNA isolation

RNA isolation from homogenates and cell culture supernatants was carried out with a commercial kit (High Pure Viral RNA Kit, Roche, Germany) according to the manufacturer's protocol. RNA samples were stored at -80 °C until use.

### 2.10. RT-PCR, ELISA, and COA

RT-PCR, ELISA, and COA tests were performed to determine the presence of the virus in homogenates and cell culture supernatants.

Transcriptor one-step RT-PCR kit (Roche, Germany) was used with WB1 5'-CCGCAACTTACTTGAGATCCATTATGC-3' and WB2 5'-CGTCTGGTTCAGATTCACCTGTAGTG-3 primers [24] for amplification of IPNV. Expected RT-PCR product size is 206 bp.

For each sample, 1 µL enzyme, 2 µL forward and reverse primers (10 µM), 10 µL buffer, and 30 µL distilled water were combined, and 5 µL of RNA sample was added. Cycling conditions were as follows: Incubation with reverse transcriptase at 50 °C for 30 min, inhibition of reverse transcriptase at 95 °C for 15 min followed by 35 cycles at 94 °C for 30 s, 52 °C for 30 s, 68 °C for 1 min with a final extension at 68 °C for 1 min. The PCR products were run in agarose gel (1.5%) containing ethidium bromide at 80V

for 1 h (Thermo, 4000P Power supply, USA) and finally were visualized under UV (Vilber Lourmat, France).

A commercial ELISA kit (Bio-X, Belgium) was used according to the manufacturer's protocol.

Coagglutination test: One drop (25 µL) of conjugate solution was placed on the slide and one drop (25 µL) of sample was added to the conjugate solution. The mixture was incubated for 30 min at room temperature with gentle stirring.

## 3. Results

### 3.1. TCID<sub>50</sub> titres of viruses, final quantities of purified viruses, and protein

The calculated TCID<sub>50</sub>/mL<sup>-1</sup> titres of the cultivated IPNV, Sp, Ab, WB, VHS, IHN and EHN viruses were 10<sup>7.25</sup>, 10<sup>7.25</sup>, 10<sup>9.75</sup>, 10<sup>7.75</sup>, 10<sup>7.75</sup>, and 10<sup>7.75</sup>, respectively. For immunization, 2400 mL Sp, 2700 mL WB, and 3000 mL Ab virus suspensions were purified and 2985 µg, 2546 µg, and 3519 µg viral proteins were obtained, respectively.

### 3.2. SN<sub>50</sub> titre of produced antisera

At the end of the microneutralisation assay, cross reactions were observed among the IPNV serotypes while neutralisation did not occur between IPNV viruses and other viruses (VHSV, IHNV and EHN). SN<sub>50</sub> titres vs. 100TCID<sub>50</sub> of antisera are provided in Table 3.

### 3.3. Optimal sera and S. aureus quantities

While a reaction was not observed when used conjugate which was prepared with 1/200 diluted S. aureus, weak positivity was observed when using conjugate prepared with 1/100 diluted S. aureus. Nonspecific reactions were seen despite strong positivity when using conjugate prepared with the use of 1:1 (v/v) antisera and S. aureus. On the other hand, strong positivity without nonspecific reactions was achieved when using conjugate prepared with S. aureus (1:10 dilution) and 250 µL antisera. The antisera and S. aureus ratio used to determine optimal conjugate are provided in Table 1.

### 3.4. Validation and detection limit

According to the validation results, the sensitivity and specificity of the test were determined to be 75% and 100% respectively (Table 4).

The detection limit of the COA test was found to be  $10^{5.25}$  TCID<sub>50</sub>/mL for Sp and Ab serotypes and  $10^{7.75}$  TCID<sub>50</sub>/mL for the WB serotype.

### 3.5. Virus isolation, RT-PCR, ELISA, and COA test results

CPE was observed in 30 out of 90 samples at the end of the third passage. RT-PCR, ELISA, and COA were performed with fish sample homogenates and CPE positive cell culture supernatants. No positive reaction was observed with ELISA and the COA test while there were 4 positive reactions with RT-PCR in homogenates. All CPE positive cell culture supernatants were found to be positive with RT-PCR, ELISA, and COA (Figures 1 and 2).

## 4. Discussion

Antigen preparation, animal and adjuvant selection, immunization methods, and finally, antibody collection

are critical steps in polyclonal antibody production [25]. In literature, different methods and experimental animals have been used for antiIPNV polyclonal sera production. Sanjuan et al. [26] reported that the rabbits are more suitable species for polyclonal antibody production against IPNV than guinea pigs. Protein A can bind to IgG1 and IgM produced in rabbits but it binds to only IgG1 and IgG2, not IgM or IgA when antiserum is produced in guinea pigs [27]. That is why guinea pigs were preferred for antisera production in this study.

The quality and quantity of the antigen in antibody production are vitally important. The use of purified antigen increases the production of specific antibodies as well as inhibiting the production of undesirables [25,28]. Hence, purified antigens were used for antibody production in this study.

It is reported that using Freund's complete adjuvant (FCA) leads to higher levels of cross reaction than FIA for antibody production [26]. Similarly, Kalaycı et al. [9] reported that the antiserum produced using FCA in rabbits had a higher cross-reaction ratio and serum neutralisation

Table 3. SN<sub>50</sub> titres of antisera.

	SN <sub>50</sub> titres					
	IPNV Sp	IPNV WB	IPNV Ab	IHNV	EHNV	VHSV
IPN (Sp) antisera	1/16000	1/2850	1/2550	0	0	0
IPN (WB) antisera	1/1190	1/23000	1/2426	0	0	0
IPN (Ab) antisera	1/2350	1/1650	1/8192	0	0	0

SN: Serum Neutralization, IPNV: Infectious Pancreatic Necrosis Virus, Sp, WB and Ab: Serotypes of IPNV, IHNV: Infectious Hematopoietic Necrosis Virus, EHNV: Epizootic Haematopoietic Necrosis Virus, VHSV: Viral Haemorrhagic Septicaemia Virus

Table 4. Validation test results.

	Staff		Verification	
	Person-1	Person-2	True results	False results
Positive	20	20	30	0
Negative	40	40	30	10
Total Samples	60	60	60	10
	Results			
	Positive		Negative	Total
Positive	TP (30)		FN (10)	TP+FN (40)
Negative	FP (0)		TN (30)	FP+TN (30)
Total	TP+FP (30)		FN+TN (40)	N

TP: True Positive, FN: False Negative, FP: False Positive, TN: True Negative

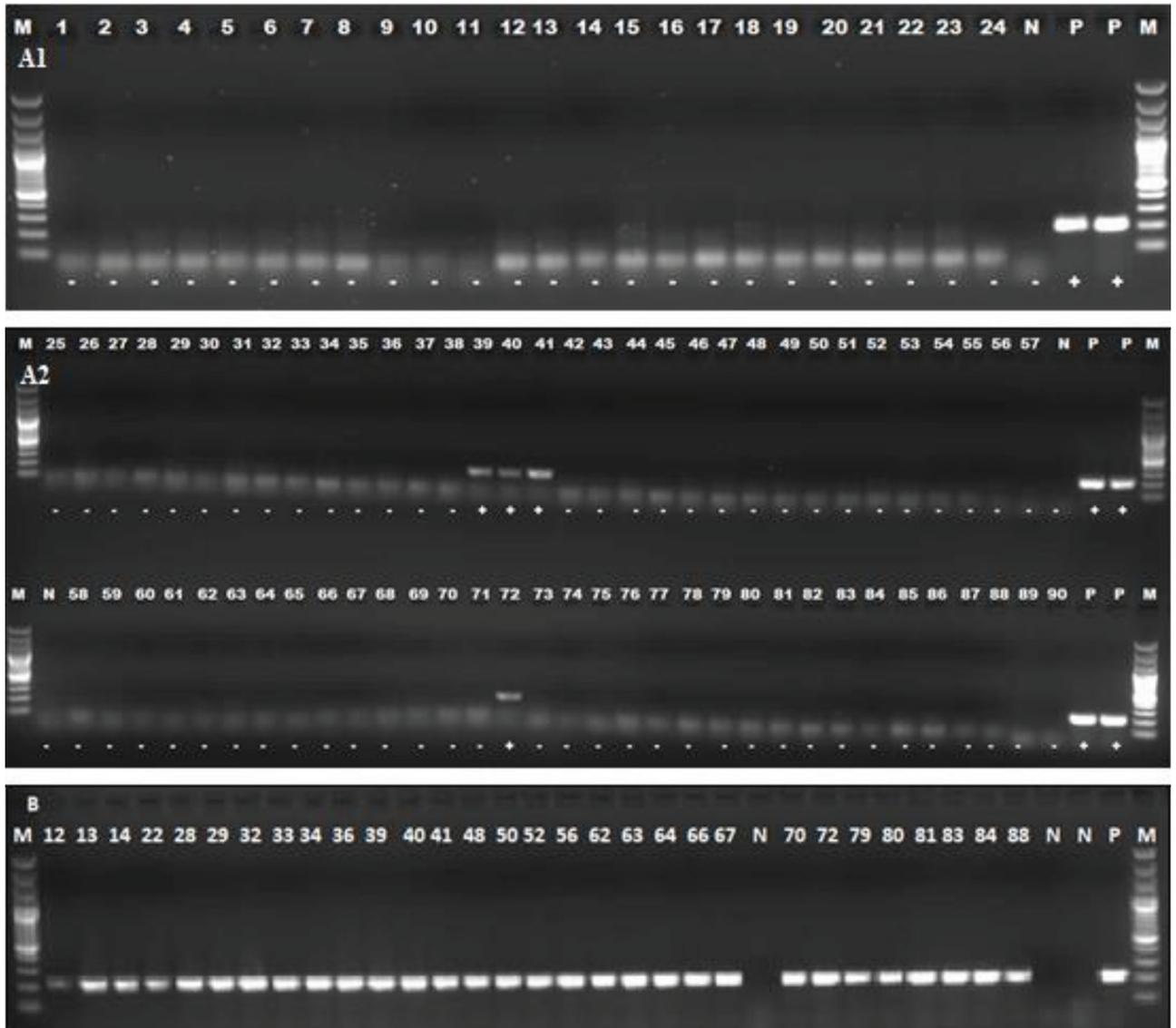


Figure 1. RT-PCR results of 90 homogenate (A1 and A2) and RT-PCR results of 30 CPE positive supernatants (B).

titres than FIA. Researchers indicated that  $SN_{50}$  titres were 1/1585, 1/168, and 1/200 for WB, Ab, and Sp, respectively, in their study based on antibody production using FCA against IPNV serotypes [19]. Although FCA was used in the study carried out by Özkan Özyer and Çağırğan [19],  $SN_{50}$  titres were lower than our results. However, it should be considered that the quantities of antigen, and immunization protocols were not the same in those two studies.

It was determined that the conjugate prepared with 2 mL of *S. aureus* (1:10 dilution) and 250  $\mu$ L antiserum was the best composition in this study. While positive reaction was not observed in the conjugates prepared to 1:200 dilution of *S. aureus*, weak positivity was observed with 1:100 dilution. Despite strong positivity nonspecific reactions

were observed when using conjugate prepared with 1:1 (v/v) serum and *S. aureus*. The same results were previously reported by other authors who mention that using high amounts of *S. aureus* and/or antiserum in conjugate causes nonspecific reactions [29–31].

Conjugate prepared with unpurified serum did not cause any reaction; the test could only be conducted after antiserum purification. It is thought that this reaction failure could be due to the salt and/or inhibitor content of the sera.

Kimura et al. [21] reported that the minimum detection limit was  $10^{5.9}$ – $10^{7.7}$  TCID<sub>50</sub>/mL for the COA test. Similarly, it was found to be  $10^5$  TCID<sub>50</sub>/mL in a previously study [32]. Our detection limits were found to be  $10^{5.25}$  for Sp and

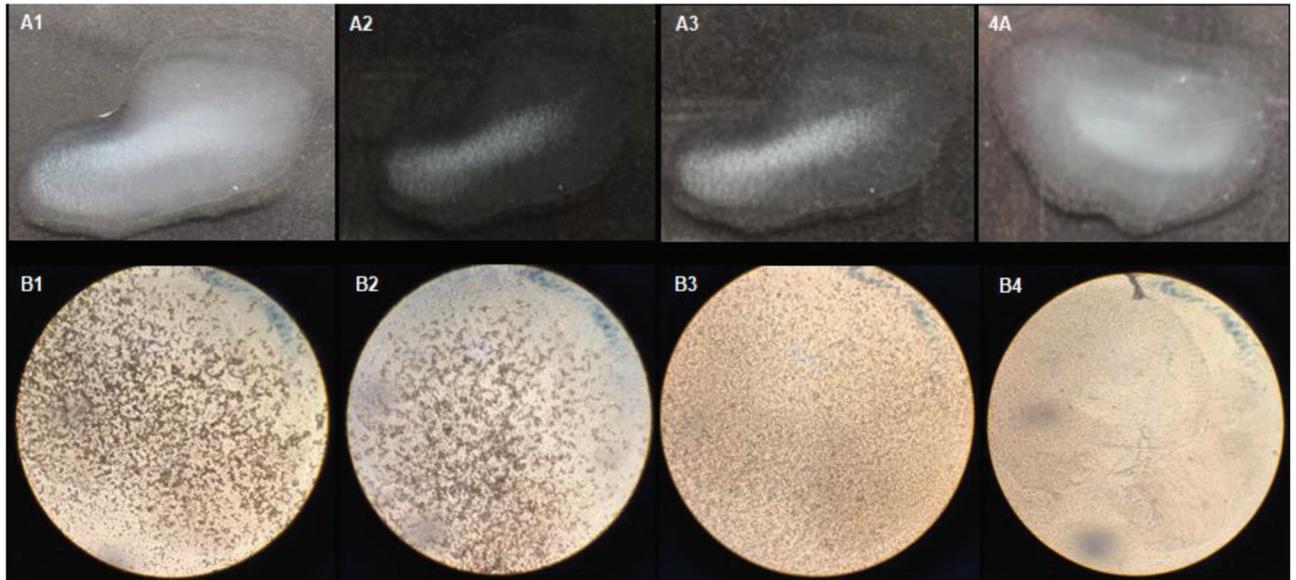


Figure 2. Coagglutination test results: Line A, naked eye inspection; line B microscopic appearance. A1-3 and B1-3 COA positive; A4 and B4 COA negative.

Ab and  $10^{7.75}$  TCID<sub>50</sub>/mL for WB and are consistent with previous studies [21,32].

In this study, IPNV positive homogenates were not detected by COA while positive cell culture supernatants were positive with COA, ELISA, and RT-PCR. According to Taksdal and Thourd [32], the COA test cannot detect IPNV from homogenates of asymptomatic carrier fish while it can detect IPNV in clinically infected fish. Similarly, because samples used in this study were obtained from asymptomatic carrier fish, IPNV couldn't be detected from homogenates. According to the validation results of this study, specificity was 100% while sensitivity was 75%. Nevertheless, if CPE-positive cell culture supernatant was selected as the test material, sensitivity increases to 100%. Both literature and the results of this study show that although the reliability of positive results is high,

the reliability of negative results is low due to the limited detection of COA.

In conclusion, the COA test can be used confidently after virus isolation in cell culture for virus detection. However, the samples obtained from asymptomatic carrier fish should be inoculated in cell culture and then tested with COA to detect IPNV.

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#### Conflict of interest

The authors declare no conflict of interest.

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