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Rapid, Simple Serological Diagnosis of Infectious Pancreatic Necrosis by Coagglutination Test Using Antibody-Sensitized Staphylococci

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The specificity and rapidity of coagglutination test for the serological typing of IPNV isolated in cell cultures or for the direct detection of IPNV antigen in fish tissues were studied.

Coagglutination tests were carried out with cell free virus antigens from selected viruses using *Staphylococcus aureus* (Cowan I) sensitized with anti-IPNV (Buhl) serum. Positive reactions occured only with cell free IPNV (Buhl) antigen, and no cross reactions were observed.

The specificity of coagglutination test for rapid (less than 2 h) serological typing of IPNV was tested using the strains from North America, Japan and Europe. Staphylococci sensitized with anti-VR 299 serum gave positive reactions with the strains from North America and Japan (VR 299 type), but did not combine with the strains from Europe (Sp and Ab type). Although staphylococci sensitized with anti-Sp and Ab sera showed weak cross reactions with the strains from North America and Japan, the agglutination pattern was clearly different.

The most important use of the coagglutination test for IPNV would be to detect specific IPNV antigen in the internal organs of infected fish. It was found that IPNV antigen could be detected in extracts of IPNV infected rainbow trout (*Salmo gairdneri*), coho salmon (*Oncorhynchus kisutch*) and amago salmon (*Oncorhynchus rhodurus*) tissues at relatively high sensitivity.

The results obtained in this study using the coagglutination test for the diagnosis of IPN indicate that this rapid and simple test, which requires no special apparatus and can be performed in the field, is a valuable addition to the diagnostic methods available for detecting this disease.

Infectious pancreatic necrosis (IPN) and infectious hematopoietic necrosis (IHN) are major disease problems for the rainbow trout culture in Japan. Epizootics caused by these viruses may persist for several weeks and the cumulative mortality may be high.

In suspected cases of viral disease it is essential that the diagnosis is made quickly and accurately so that effective control measures may be introduced with a minimum of delay. Although the clinical signs and histopathological features of these viral diseases may permit a presumptive diagnosis, confirmation is required by identification of the specific virus in the diseased tissues. The most commonly used confirmatory method is isolation of the virus in fish cell cultures followed by neutralization test with specific antiserum. This procedure often requires one to two weeks for completion.

Several methods for the rapid diagnosis of these diseases have been reported. These include the fluorescent antibody test (FAT) (PIPER, NICHOLSON and DUNN 1973, JORGENSEN 1974, TU, SPENDLOVE and GOEDE 1975, HEDRICK, LEONG and FRYER 1978, SANO and NISHIMURA 1981), immunoperoxidase staining (IP) (NICHOLSON and HENCHAL 1978, SANO and NISHIMURA 1981) and enzyme-linked immunosorbent assay (ELISA) (NICHOLSON and CASWELL 1982). However, most of these methods still require virus isolation in fish cell culture. A more rapid confirmatory diagnosis can be achieved by direct serological demonstration of the viral antigen in the tissues of diseased fish. Reports of above direct approach are limited to the use of FAT (SWANSON and GILLESPIE 1981) and ELISA (DIXON and HILL 1983). Moreover, these virus identification methods have not been widely adopted for routine use by fish culturists or pathologists because special techniques and equipment were required.

We have reported that the coagglutination test using antibody-sensitized staphylococci was a

rapid and reliable diagnostic method for bacterial kidney disease, vibriosis and furunculosis (KIMURA and YOSHIMIZU 1981, 1982, 1983a, 1984). Recently, RAJAGOPLAN and JACOB-John (1982) reported that the coagglutination test was useful for detection of hepatitis B virus surface antigen and that the sensitivity of this method was 30 times greater than counterimmunoelectrophoresis (CIE). KATZ, STRAUSSMAN, SHAHAR and KOHN (1980) and NICOLAIEFF, KATZ and REGENMORTES (1982) have reported that solid phase immune electron microscopy (SPIEM) using antibody-sensitized staphylococci, was applied to the detection of sindbis virus and plant viruses. We have reported that the coagglutination and SPIEM techniques were useful for rapid serological identification of IPNV isolated in cell culture (KIMURA and YOSHIMIZU 1983b).

The purpose of this study was to determine the specificity and rapidity of the coagglutination test for the serological typing of IPNV isolated in cell cultures or for the direct detection of IPNV antigen in fish tissues. The simplicity and reliability of this technique would allow it to be used for routine field diagnosis.

Materials and Methods

Antisera:

Four rabbit antisera against strains of IPNV; VR 299 (ATCC), Sp, Ab and Buhl were used in this study. The anti-Buhl serum (50% neutralization titer $10^{4.3}$ vs 100 $TCID_{50}$) was provided by Dr. K. WOLF, National Fish Health Research Laboratory, Leetown, West Virginia, U.S.A., and anti-VR 299, Ab and Sp sera (50% neutralization tiers $10^{5.5}$, $10^{5.7}$ and $10^{5.7}$ vs 100 $TCID_{50}$) were provided by Dr. R. P. HEDRICK, Bodega Marine Laboratory, University of California, U.S.A. Antiserum for IHNV was prepared against the Yurappu strain isolated from chum salmon (*Oncorhynchus keta*) in Hokkaido. *Oncorhynchus masou* virus (OMV) antiserum was prepared against the Otobe strain OO-7812 (50% neutralization titer of above both sera was $10^{2.0}$). All antisera were absorbed before use with an equal volume of fetal bovine serum (FBS) containing acetone powdered RTG-2 cells (50 mg/m*l*). The mixture was incubated at 37°C for 2 h and then at 5°C overnight to avoid nonspecific reactions.

Preparation of stabilized staphylococci:

Staphylococcus aureus ATCC 12598 (Cowan I) strain which is rich in protain A was used to bind antibody.

Stabilization of staphylococci was carried out by the method of KIMURA and YOSHIMIZU (1981). Briefly, *Staphylococcus aureus* was cultured overnight in trypticase soy broth, the cells were washed five times in phosphate buffered saline (PBS, pH 7.3) and resuspended in 0.5% formalin-PBS. After 3 h at 25°C, the cells were washed three times in PBS and reconstituted with PBS to a 10% (v/v) suspension. This suspension was heated at 80°C for 1 h, washed three times with PBS, made up to a 10% (v/v) suspension with PBS containing 0.1% sodium azide and then stored at 4°C.

Coupling of staphylococci and antibody:

To 1 m*l* of the stabilized cell suspension of staphylococci, 0.1 m*l* of antiserum was added, and the mixture incubated at 25°C for 3 h following. During incubation, the mixture was gently shaken every 30 min, subsequently centrifuged at 5°C for 60 min ($2810 \times g$), and the pellet of sensitized staphylococci was resuspended in PBS at a concentration of 0.5% (v/v) for use.

Cell culture:

Monolayer cultures of rainbow trout (*Salmo gairdneri*) gonad (RTG-2) cells were grown in EAGLE's minimal essential medium (MEM) containing 10% fetal bovine serum (MEM-10) with 100 IU of penicillin and 100 μg of streptomysin per m*l*.

Virus:

Stock virus suspension of 19 IPNV isolates from Japan, North America and Europe were prepared in RTG-2 cell cultures. North American isolates included ATCC reference strain VR 299, Buhl (Idaho), West Buxton (Maine), Dry Mills (Maine) and Reno (Nev.). European isolates included Sp (Denmark)(Bonnamy (France), d'Honninchton (France) and Ab (Denmark). Japanese isolates included six strains from rainbow trout: Nichiro, Matsuhisa, Eniwa, Saito, Yamamoto and Gifu, and four strains from pacific salmon: Oippe and Towada strains isolated from masu salmon (*Oncorhyncus masou*), Gifu strains isolated from coho salmon (*Oncorhyncus kisutch*) and amago

salmon (*Oncorhyncus rhodurus*). And also, to compare with others than IPNV, rhabdovirus (IHNV, Yurappu strain isolated from chum salmon in Hokkaido) and herpesvirus controls (OMV, OO-7812 from masu salmon in Hokkaido) were used in this study. Cell free virus antigen was prepared by filtration through a 0.45 μ membrane filter (Millipore HA).

Fish specimens:

Thirty six fry is a total, six salmonid species suspected of having IPN were sampled at random from five fish farms located in Yamanashi and Gifu Prefecture. These specimens were mailed to the laboratory in ice packed condition except three specimens of coho salmon (No. G-4 to 6) which were received in dry ice. Control fish consisted of three healthy chum salmon which were cultured in our laboratory. Specimens were tested immediately upon arrival.

Preparation of fish extract antigens:

Virus antigen was prepared from fish whole viscera as a tissue homogenate (5% w/v in HANKS' BSS). The homogenate was sterilized by membrane filtration for use as a viral antigen or for virus isolation.

Coagglutination test:

A volume of 0.05 m*l* of antibody-sensitized staphylococci suspension and 0.05 m*l* of the test antigen was mixed on a glass slide, and allowed to react in a moist chamber for 30, 60 and 90 min. At the end of each incubation period, the glass slide was examined by visual inspection or with a microscope ($\times 10$) for development of the coagglutination reaction.

Results

Specificity of the coagglutination reaction for identification of IPNV cultured in RTG-2 cells:

Cross coagglutination tests were carried out using rabbit antisera against IPNV (Buhl), IHNV (Yurappu) and OMV (OO-7812). These were tested against cell free virus antigens, virus free culture medium from RTG-2 cells, MEM-10 and FBS. Staphylococci sensitized with unabsorbed antisera showed positive reactions with all antigens employed, but staphylococci sensitized with antisera absorbed with FBS and acetone powdered RTG-2 cells gave positive reactions only when anti-IPNV rabbit serum sensitized staphylococci and a cell free IPNV antigen were used. Heterologous antigens and staphylococci sensitized by normal rabbit serum gave negative results. Anti-IHNV and OMV rabbit sera sensitized staphylococci showed no reaction with either homologous or heterologous antigens (Table 1).

The specificity of the reaction was confirmed with a blocking test (Table 2). The blocking test was carried out using staphylococci sensitized with antiserum against IPNV (Buhl) and a cell free IPNV (Buhl) antigen which had been mixed with anti-IPNV (Buhl) rabbit serum for 1 h at room temperature. A positive reaction only occurred when the anti-IPNV sensitized staphy-

Table 1. Cross coagglutination tests using staphylococci sensitized with IPNV (Buhl), IHNV (HV–7601) and OMV (OO–7812) antisera against selected cell culture and viral antigens

Antigen used	Antisera used to sensitized staphylococci						
	Anti-IPNV Buhl		Anti-IHNV Yurappu		Anti-OMV OO–7812		Control*1
	A*2	B*3	A	B	A	B	A
Fetal Bovine Serum	+	−	+	−	+	−	−
Minimal Essential Medium (MEM–10)	+	−	+	−	+	−	−
Medium from RTG-2 cell culture	+	−	+	−	+	−	−
Cell free IPNV (Buhl)	+	+	+	−	+	−	−
Cell free IHNV (Yurappu)	+	−	+	−	+	−	−
Cell free OMV (OO-7812)	+	−	+	−	+	−	−

*1 Normal rabbit serum.

*2 Unabsorbed antiserum.

*3 Antiserum absorbed with FBS and acetone powdered RTG-2 cell.

Table 2. Blocking effect of IPNV (Buhl) antiserum on the coagglutination test using staphylococci sensitized with IPNV (Buhl) antiserum and cell free IPNV (Buhl) antigen

Virus antigen used	Antisera used to sensitized staphylococci	
	Anti-IPNV (Buhl)	Control*1
Cell free IPNV (Buhl)	+	−
Cell free IPNV (Buhl) after 1 h incubation with anti-IPNV (Buhl) rabbit sera	−	−

*1 Normal rabbit serum.

lococci and unblocked IPNV antigen were used.

The coagglutination test for serological typing of cell fee IPNV cultured in RTG-2 cells:

For serological typing of cell grown IPNV antigens the specificity of the coagglutination test was evaluated using staphylococci sensitized with antisera against strains VR 299, Sp and Ab. Three different sensitized staphylococci were tested against five IPNV strains grown in cell culture from North America, ten strains from Japan and four strains from Europe (Table 3). The staphylococci sensitized with anti-VR 299 serum showed positive reactions with the strains from North America and Japan but not with the European strains. Staphylococci sensitized with anti-Sp or Ab sera showed positive reactions with only the strains from Europe after 30 min incubation. However, weak cross reactions were observed with the strains from North America and Japan after incubation periods exceeding 1 h. The agglutinating particles were very fine and different from those in the reaction with the Sp and Ab strains (Fig. 1). These results indicated the usefulness of the coagglutination test for rapid serological typing of IPNV for the North American and European strains. The minimum amount of viral antigen needed to get a positive reaction varied between $10^{5.9}$ and $10^{7.7}$ $TCID_{50}/ml$.

Effect of antigen concentration and time of reaction on the coagglutination test for detection of IPNV antigens in fish tissue extracts:

Antigen extracts from fish were prepared as a

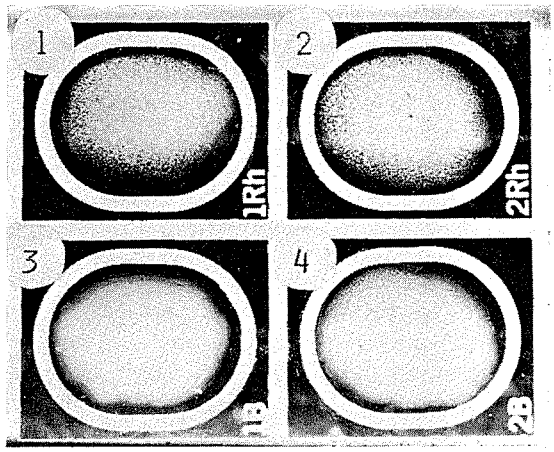

Fig. 1. Coagglutination reaction patterns obtained with MEM_{10} (3), cell free IPNV strain VR 299 (4), Sp (1) and Ab (2), using a staphylococcal suspension sensitized with anti-IPNV (Strain Sp) rabbit serum.

1 : 5 tissue suspension (w/v) in HANKS' BSS using whole viscera of IPN infected rainbow trout, coho salmon and amago salmon. Two-fold dilutions of these extracts in BSS from 1 : 5 to 1 : 320 were used. All antigens were tested against staphylococci sensitized with IPNV(Buhl)antiserum. A positive reaction occurred with antigens from IPNV infected fish and the more concentrated dilutions tended to give positive reactions with 30 min, while the higher dilutions required a longer time. A control antigen prepared from healthy chum salmon failed to react at any concentration tested. The staphylococci sensitized with normal rabbit serum also failed to react with any of the antigen preparations (Table 4). These experiments demonstrated the ability of the coagglutination test to detect the IPNV antigen at relatively high dilutions of fish tissue within 30 min.

Diagnosis of IPN in fish undergoing natural epizootics using the coagglutination test:

A total of 36 fish specimens consisting of six salmonid species were collected at random from five fish farms in Gifu and Yamanashi Prefecture. All fishes seemed to be experiencing an epizootic of IPN. Three healthy chum salmon fry were used for controls. All fishes were examined for evidence of IPN by the coagglutination test. Samples were also processed for the isolation and titration

Table 3. Specificity of coagglutination tests using staphylococci sensitized with anti-IPNV rabbit serum

IPNV antigen used		Antisera used to sensitize staphylococci												Minimum titer showing positive reaction ($TCID_{50}/ml$)
IPNV	Titer ($TCID_{50}/ml$)	Anti-IPNV serum									Control*1			
		VR 299			Sp			Ab						
		30*2	60	90	30	60	90	30	60	90	30	60	90	
VR 299	7.05	+	+	+	−	+	+	−	−	+	−	−	−	6.45
Buhl	7.55	+	+	+	−	+	+	−	+	+	−	−	−	6.95
West Buxton	7.80	+	+	+	−	+	+	−	−	+	−	−	−	7.20
Dry Mills	7.80	+	+	+	−	+	+	−	+	+	−	−	−	7.50
Reno	7.55	+	+	+	−	+	+	−	+	+	−	−	−	6.95
Nichiro	7.05	+	+	+	−	+	+	−	−	+	−	−	−	6.45
Matsuhisa	6.80	+	+	+	−	+	+	−	−	+	−	−	−	6.50
Oippe	8.30	+	+	+	−	+	+	−	+	+	−	−	−	7.70
Eniwa	8.30	+	+	+	−	+	+	−	+	+	−	−	−	7.70
Yamamoto	6.80	+	+	+	−	+	+	−	−	+	−	−	−	5.90
Gifu RT	7.55	+	+	+	−	−	+	−	−	+	−	−	−	6.95
Gifu CO	7.05	+	+	+	−	−	+	−	+	+	−	−	−	6.45
Gifu AM	7.05	+	+	+	−	+	+	−	+	+	−	−	−	6.45
Towada	7.80	+	+	+	−	−	+	−	+	+	−	−	−	7.50
Saito	7.05	+	+	+	−	+	+	−	+	+	−	−	−	6.75
Sp (Denmark)	8.55	−	−	−	+	+	+	+	+	+	−	−	−	7.65
Bonnamy (France)	8.30	−	−	−	+	+	+	+	+	+	−	−	−	7.70
d'Honninchton (France)	8.80	−	−	−	+	+	+	+	+	+	−	−	−	7.63
Ab (Denmark)	8.30	−	−	−	+	+	+	+	+	+	−	−	−	7.40

*1 Normal rabbit serum.
*2 Reaction time.

Table 4. Effect of concentration and the time of reaction on the coagglutination test*3 for detection of IPNV antigen in extracts of fish collected from Gifu Prefecture

Concentration of tissue	Rainbow trout No. R-1			Coho salmon No. C-1			Amago salmon No. A-1			Chum salmon*1 No. Ch-1		
	30*2	60	90	30	60	90	30	60	90	30	60	90
1: 5	+	+	+	+	+	+	+	+	+	−	−	−
1: 10	+	+	+	+	+	+	+	+	+	−	−	−
1: 20	+	+	+	+	+	+	+	+	+	−	−	−
1: 40	+	+	+	+	+	+	+	+	+	−	−	−
1: 80	+	+	+	−	+	+	+	+	+	−	−	−
1: 160	−	−	−	−	−	−	−	−	−	−	−	−
1: 320	−	−	−	−	−	−	−	−	−	−	−	−

*1 Healthy fish for control.
*2 Reaction time.
*3 Anti-IPNV (Buhl)

of IPNV from individual fish (Table 5).

Staphylococci sensitized with antiserum against IPNV (Buhl) showed positive coagglutination reactions with rainbow trout, coho and amago salmon cultured in Gifu Prefecture. The virus was isolated from all fish except the coho salmon No. G-4 to 6 which were recieved by our laboratory chilled with dry ice. The antigens prepared from the rainbow trout cultured in Yamanashi Prefecture, from which IPNV was not isolated, and

Table 5. Comparison of coagglutination test and isolation of IPNV for detection of IPN disease

Fish No.	Species of fish	Source of fish	Virus		Coagglutination test*2	Minimum titer showing positive reaction
			Isolation	Titer*1		
R-1	Rainbow trout	Gifu	+	9.05	+	7.15
R-2	Rainbow trout	Gifu	+	7.80	+	6.50
Co-1	Coho salmon	Gifu	+	5.00	+	3.10
Co-2	Coho salmon	Gifu	+	5.03	+	3.35
Co-3	Coho salmon	Gifu	+	5.75	+	3.85
Co-4*3	Coho salmon	Gifu	−	<4.50	+	ND*4
Co-5*3	Coho salmon	Gifu	−	<4.50	+	ND
Co-6*3	Coho salmon	Gifu	−	<4.50	+	ND
A-1	Amago salmon	Gifu	+	5.25	+	3.05
A-2	Amago salmon	Gifu	+	5.78	+	4.40
A-3	Amago salmon	Gifu	+	4.75	+	3.15
R-13 to R-17	Rainbow trout	Yamanashi	−	ND	−	ND
R-18 to R-27	Rainbow trout	Yamanashi	−	ND	−	ND
Ch-1	Chum salmon	Laboratory	−	ND	−	ND
Ch-2	Chum salmon	Laboratory	−	ND	−	ND
Ch-3	Chum salmon	Laboratory	−	ND	−	ND

*1 $TCID_{50}$/g whole viscera.
*2 Staphylococci sensitized with antisera for IPNV (Buhl).
*3 Mailed with dry ice.
*4 Not determined.

the control antigens prepared from healthy chum salmon showed negative reactions.

Discussion

The development of a reversed passive agglutination or coagglutination test for the serological typing of pneumococci (KRONVALL 1973) and dectection of *Neisseria gonorrhoea* (DANIELSON and KRONVAL 1973) using staphylococci sensitized with specific antibody, indicated that this general method might have potential of serological applications for the diagnosis of other fish pathogens. We have reported that the coagglutination test with specific antibody-sensitized staphylococci was a rapid and reliable field diagnostic method for bacterial kidney disease, vibriosis and furunculosis of salmonid fish (KIMURA and YOSHIMIZU 1981, 1982, 1983a, 1984). The finding that staphylococci sensitized with anti-IPNV serum would bind IPNV on the surface of the cells and agglutinate indicated that this method might be useful for the diagonosis of IPNV (KIMURA and YOSHIMIZU 1983b).

In the present study, the application of this method for the diagnosis of infectious pancreatic necrosis in salmonids was investigated. Coagglutination tests were carried out with cell free virus antigens from selected viruses using staphylococci sensitized with anti-IPNV (Buhl) serum. Positive reactions occurred only with cell free IPNV (Buhl) antigen, and no cross reactions were observed.

The serological differences between North American and European IPNV isolates have been described by JORGENSEN and KEHLET (1971), UNDERWOOD, SMALE, BROWN and HILL (1977), FINLAY and HILL (1975), MACDONALD and GOWER (1981) and OKAMOTO, SANO, HEDRICK and FRYER (1983). The specificity of coagglutination test for serological typing of IPNV was tested using five strains from North America, ten strains from Japan and four from Europe. Staphylocci sensitized with anti-VR 299 serum gave positive

reactions with the strains from North America and Japan, but did not combine with the strains from Europe. Although staphylococci sensitized with anti-Sp and Ab sera showed weak cross reactions with the strains from North America and Japan after more than 1 h incubation, the agglutination pattern was clearly different. These results indicate the coagglutination test is useful for rapid serological typing of IPNV for at least two groups, the North American and European strains.

The most important use of the coagglutination test for IPNV would be to detect specific IPNV antigen in the internal organs of infected fish. It was found that IPNV antigen could be detected in extracts of IPNV infected rainbow trout, coho salmon and amago salmon tissue at relatively high dilutions.

The coagglutination test for diagnosis of IPN was evaluated. Although we did not compare the sensitivity of this test with other methods, the minimum virus titer necessary for a positive reaction was slightly higher than that required for an ELISA test (DIXON and HILL 1983). Using extracts of infected fish, the numbers of specimens found to be positive by the coagglutination test was higher than the numbers detected by virus isolation. These results suggest that the technique has sufficient sensitivity for diagnostic applications. The results obtained in this study using the coagglutination test for the diagnosis of IPN indicate that this relatively simple test, which requires no special apparatus and can be performed in the field, is a valuable addition to the diagnostic methods available for detecting this disease.

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特異抗体感作 staphylococci を用いた coagglutination test の IPN 迅速診断への応用について

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特異抗体感作 staphylococci を用いた coagglutination test による感染細胞培養液および病魚内臓抽出液中に存在する IPN ウイルス抗原の検出法について，特に本法の IPN 迅速診断への応用を目的に検討を行ない次の如き結果を得た。

抗 IPNV (Buhl) 血清感作 staphylococci は IPNV (Buhl) と反応陽性を，IHNV や OMV とは陰性を示し，この反応の特異性はブロッキングテストにより確かめられた。血清タイプの異なる IPNV 株間での交叉凝集試験では，抗 IPNV VR299 血清感作 staphylococci は北米および我国分離株 (VR299 type) とのみ反応陽性を示し，Ab, Sp 株を含むヨーロッパ株との反応は陰性であった。一方抗 IPNV Ab, Sp 血清感作 staphylococci は VR 299 type 株と弱い交叉凝集反応を示したが，その凝集パターンは Ab, Sp 株のそれとは明らかに異なり，本法によりこれらの迅速・簡易血清型別が可能であった。

ニジマス (*Salmo gairdneri*)，ギンマス (*Oncorhynchus kisutch*) およびアマゴ (*Oncorhynchus rhodurus*) IPN 病魚の内臓抽出液を抗原とした coagglutination test では，比較的高希釈 ($\times 2^4$) 試料においても 90 分間の反応で陽性結果を得，IPN の迅速な診断が可能となった。

本法は特殊な実験器具を全く必要とせず，最終結果を得るまでにわずか 2 時間程を要するのみであることから，現場における IPN の迅速かつ簡易な診断法であると考える。

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