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A Coagglutination Test with Antibody-Sensitized Staphylococci for Rapid and Simple Diagnosis of Bacterial and Viral Diseases of Fish

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The application of a coagglutination test for the diagnosis of diseases in fish was studied using staphylococci specifically sensitized with antibodies against the bacteria causing bacterial kidney disease (BKD), furunculosis, vibriosis and goldfish ulcer disease, and also against the virus causing infectious pancreatic necrosis (IPN). This method proved to be a simple, rapid and reliable diagnostic test suitable for use in the laboratory or field and requires no special apparatus.

Procedures for this method are summarized as follows:

1. The kidney or affected tissue samples from the diseased fish are homogenized in four to nine times their volume of PBS or HANKS' BSS. If the antigen is heat stable, it is also heated in a boiling water bath for 30 min.
2. The supernatant material is collected after centrifugation at 4000 rpm for 20 min. This may be omitted if a centrifuge is unavailable.
3. One drop of the supernatant material and one drop of antibody-sensitized staphylococci suspension are mixed on a glass slide and incubated in a wet chamber at room temperature. The slide is examined after 30, 60 and 90 min.
4. If coagglutination is observed, the infected fish should be examined using another method to confirm the diagnostic results.

Introduction

The Fc fraction of IgG molecules can be bound to protein A of a strain of *Staphylococcus aureus* (COWAN I) without blocking the specific antigen binding activity of the immunoglobulin (FORSGREEN and SJOQUIST, 1966). Reverse passive agglutination, or coagglutination tests, using such specifically sensitized staphylococci have been developed for serological typing of pneumococci and β-hemolytic streptococci (KRONVAL, 1972; EDWARD and LARSON, 1974) and for the rapid detection of *Neisseria gonorrhoeae* and *Haemophilus influenzae* antigens and also the hepatitis B virus surface antigen. (CHRISTENSEN *et al.*, 1973; SUKSNONG and DAJANI, 1977; RAJAGOPLAN and JACOB-JOHN, 1982).

It was the purpose of the present study to sensitize staphylococci of the COWAN I strain by binding specific antibody to them. These antibodies were against the bacteria causing bacterial kidney disease (EARP *et al.*, 1953), furunculosis (GRIFFIN *et al.*, 1953), vibriosis (EGUSA, 1978) and goldfish ulcer disease (carp erythrodermatitis) (ELLIOT and SHOTTS 1980; BOOTSMA *et al.*, 1977) and the virus causing infectious pancreatic necrosis (WOLF *et al.*, 1960). It was then determined whether these preparations could be used in coagglutination tests to detect the presence of specific antigens in extracts of kidney tissue or affected tissue of fish. If such a test were found to be successful and practical it could then be evaluated as a possible method for diagnosis of these diseases, and compared with other available diagnostic methods, *i.e.* the Gram stain and fluorescent antibody testing of direct kidney smears from diseased fish and the isolation of the causative bacteria or virus.

Materials and Methods

Staphylococcus aureus Rich in Protein A

Staphylococcus aureus ATCC 12598 (COWAN I) was the strain of the organism, rich in protein A, used to bind antibody.

Antisera

Sixteen rabbit antisera against the bacteria causing bacterial kidney disease (BKD), furunculosis, vibriosis and goldfish ulcer disease, and also against the virus causing infectious pancreatic necrosis (IPN) were prepared as previously described (KIMURA and YOSHIMIZU, 1981a,b, 1982, 1983a,b, 1984a,b,c).

Fish Specimens

A total of 929 fish of 6 salmonid species in addition to 33 ayu (*Plecoglossus altivelis*) and 16 goldfish (*Carassius auratus*) were sampled at random from fish farms with histories suggesting the presence of BKD, furunculosis, vibriosis, goldfish ulcer disease, or IPN. Ten rainbow trout (*Salmo gairdneri*) and 85 chum salmon (*Oncorhynchus keta*) were artificially infected with *Aeromonas salmonicida* and *Vibrio anguillarum* in our laboratory. Controls were 17 healthy masu salmon (*O. masou*), 3 chum salmon and 5 crussian carp (*C. carasius*). Some of the specimens were mailed on dry ice and stored at −20°C or −80°C prior to testing.

Preparation of Stabilized Staphylococci

Stabilization of staphylococci was carried out by the method previously described (KIMURA and YOSHIMIZU, 1981b). *Staphylococcus aureus* ATCC 12598 was cultured overnight in trypticase soy broth. The cells were harvested by centrifugation (2810 × *g*, 20 min) and washed five times with phosphate buffered saline (PBS, pH 7.2) and then resuspended in 0.5% formalin-PBS (v/v). After incubation for 3 hr at 25°C, the cells were washed three times with PBS, and resuspended in PBS at a concentration of 10% (v/v). The suspension was then heated at 80°C for 1 hr, washed three times with PBS, and the final 10% suspension (v/v) in PBS was designated as a stabilized suspension of staphylococci, ready for further treatment.

Coupling of Staphylococci and Antibody

To 1 m*l* of the above stabilized cell suspension of staphylococci, 0.1 m*l* of antiserum was added, and the reaction carried out at 25°C for 3 hr following thorough mixing. During the action, the tube was gently shaken every 30 min. The mixture was then centrifuged at 5°C for 60 min (2810 × *g*) and the pellet resuspended in PBS at a concentration of 0.5% (v/v) to be used as sensitized staphylococci.

Preparation of Antigens of Test Bacteria

All test bacteria were cultured on nutrient agar medium for 48 hr at 25°C, except that *Renibacterium salmoninarum* was cultured for 3 weeks at 15°C on KDM-2 (EVELYN, 1977). After harvest, the cells were weighed and a 10% suspension (w/v) was made in PBS. The suspension was heated at 100°C for 30 min, and then centrifuged. The resultant supernatant fluid was designated as heat-extracted antigen and the pellet was resuspended with PBS (10% w/v) to be stored as a heat-treated cell suspension antigen.

Cell Culture

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

Virus

Stock virus suspension of 19 IPNV isolates from Japan, North America and Europe were prepared in RTG-2 cell cultures. A rhabdovirus, infectious pancreatic necrosis (AMEND *et al.*, 1969) virus (IHNV), and a herpesvirus, *Oncorhynchus masou* virus (OMV; KIMURA *et al.*, 1981), were used negative controls in this study. Cell-free viral antigens were prepared by filtration through a 0.45 μ membrane filter.

Preparation of Fish Extract Antigens

The kidneys or affected tissue samples from the diseased fish were homogenized in four to nine times their volume of PBS or HANKS' BSS. When the antigen was heat stable, it was heated in a boiling water bath for 30 min, centrifuged and the supernatant fluid was recovered as the extracted antigen.

Evaluation of Antibody Coupling Ability of the Stabilized Staphylococci

One m*l* of stabilized staphylococci suspension was mixed with 0.1 m*l* of the antisera. After 3 hr of incubation at 25°C, the supernatant fluids of the reaction mixtures were separated by centrifugation (2800 × *g*, 60 min) and the antibody titers remaining against the corresponding antigens were determined. Efficacy of antibody absorption was evaluated by the reduction of the agglutinating antibody titers of the antiserum after the reaction with

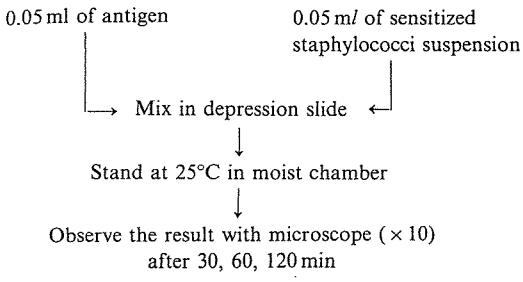

Fig. 1. Flow chart outlining the coagglutination test.

staphylococci.

The Coagglutination Test

A volume of 0.05 ml of antibody-sensitized staphylococci suspension and 0.05 ml of the test antigen were mixed on a glass slide, and the reaction was carried out in a moist chamber for 30, 60, or 120 min. At the end of the incubation period, the slide was examined by naked eye or under a microscope (× 10) for development of the coagglutination reaction. The overall reaction procedure is summarized in Fig. 1.

Immunodiffusion Test and Fluorescent Antibody Test

The immunodiffusion tests were carried out by the ordinary micro-OUCHTERLONY method (OUCHTERLONY and NILSSON, 1973). The BKD fluorescent antibody tests were carried out by the direct method using FITC conjugated anti-*R. salmoninarum* rabbit serum provided by C. BANNER, Oregon State University. For the vibrios, the indirect method was used (MCDANIEL, 1975).

Results and Discussion

Antibody Binding Ability of Stabilized Staphylococci

The antibody binding ability of the stabilized staphylococci was tested using rabbit antisera against *R. salmoninarum* and *A. salmonicida*. Agglutinating antibody titers of these antisera were markedly reduced after incubation with the stabilized staphylococci (Table 1), indicating that most of the antibody had apparently been coupled to the protein A of these organisms.

Specificity of the Coagglutination Reaction for Detection of Bacterial and Viral Antigens

Cross coagglutination tests were carried out with rabbit antisera against *R. salmoninarum*, *A. salmonicida* and *V. anguillarum*, and the corresponding heat-extracted and heat-treated cell suspension antigens. The results are shown in Table 2. Coagglutination reaction patterns are shown in Figs. 2 and 3. It was apparent that agglutination occurred only with specific combinations of antibody sensitized staphylococci and the antigen homologous for the antibody, and not with heterologous antigens or with staphylococci sensitized with normal rabbit serum. The results obtained with heat treated cell antigens were the same as those observed with heat extracted antigens, although reactions with the cell antigens were more rapid and produced larger aggregations than when heat extracted antigens were used.

Specificity was further tested with staphylococci sensitized with the same 5 antibacterial sera. These preparations were tested for possible reactions against heat extracted antigens of *Aeromonas*

Table 1. Antibody binding ability of stabilized staphylococci

Antiserum	Agglutinin titer of antiserum before and after absorption with staphylococci		Percent of antibody bound
	Before	After	
Anti-*Aeromonas salmonicida* ATCC 14174	25,600	400	98.4
Anti-BKD bacterium*[1] strain Otobe (O-1)	1,600	50	96.9

*[1] Kidney disease bacterium; *Renibacterium salmoninarum*.

Table 2. Cross coagglutination tests using staphylococci sensitized with antibody specific for each of three bacterial pathogens and antigens prepared from cells of these organisms

Bacterial antigen used*1 as heat treated cell suspension	Antisera used to sensitize staphylococci					
	Anti-BKD bacterium			Anti-*Aeromonas salmonicida* ATCC 14174	Anti-*Vibrio anguillarum* KAY-2	Control*5
	Strain EFDL-2*2	Strain AKD-3*3	Strain OKD-3*4			
BKD bacterium strain Otobe	+*6	+	+	−*6	−	−
BKD bacterium strain Erimo	+	+	+	−	−	−
Aeromonas salmonicida ATCC 14174	−	−	−	+	−	−
Vibrio anguillarum strain KAY-2	−	−	−	−	+	−

*1 Heated in PBS at 100°C for 30 min. After centrifuging the pellet resuspended in PBS was the cell suspension antigen. Supernatant fluid was the heat extract antigen. All tests were conducted with each type of antigen and results were identical in each case.
*2 Antiserum provided by National Fish Health Research Lab., Leetown W. Va., U.S.A.
*3 Antiserum against strain Marimo.
*4 Antiserum against strain Otobe.
*5 Normal rabbit serum.
*6 + indicates a positive agglutination reaction; − indicates a negative reaction.

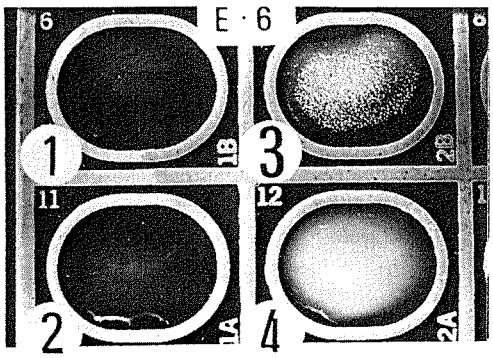

Fig. 2. Coagglutination reaction patterns obtained with heated cell suspension of BKD bacterium strain Erimo, using a staphylococcal suspension coupled with anti-BKD serum, AKD-3 (3). The same cell suspension did not agglutinate with normal rabbit serum bound staphylococci (4), and neither staphylococcal suspension agglutinated with PBS (1, 2).

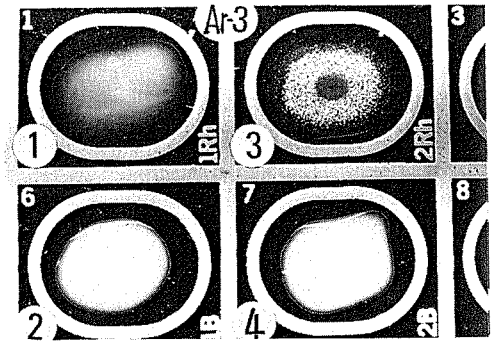

Fig. 3. Coagglutination reaction patterns obtained with heat extracted antigen of *Aeromonas salmonicida* ATCC 14174 (Ar-3), using a staphylococcal suspension coupled with anti-*A. salmonicida* ATCC 14174 serum (3). The same extracted antigen did not agglutinate with normal rabbit serum bound staphylococci (4), and neither staphylococcal suspension agglutinated with PBS (1, 2).

species, *Vibrio* species, *R. salmoninarum*, *Escherichia coli*, *Pseudomonas fluorescens*, *Micrococcus lysodeikticus*, and *Bacillus subtilis*. Once again, the only agglutination reactions observed occurred with antibody sensitized staphylococci and the antigen homologous for the antibody (Table 3). The evidence from all of these tests indicates a high degree of specificity for this coagglutination reaction.

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.

Table 3. Specificity of coagglutination tests using staphylococci sensitized with antibody specific for each of three bacterial pathogens

Bacterial antigen used*1	Antisera used to sensitize staphylococci					
	Anti-BKD bacterium			Anti-*Aeromonas salmonicida* ATCC 14174	Anti-*Vibrio anguillarum* KAY-2	Control*5
	Strain EFDL-2*2	Strain AKD-3*3	Strain OKD-3*4			
BKD bacterium strain Erimo	+*6	+	+	−*6	−	−
BKD bacterium strain Otobe	+	+	+	−	−	−
BKD bacterium strain Niigata	+	+	+	−	−	−
BKD bacterium strain Shizuoka	+	+	+	−	−	−
BKD bacterium strain Miyako	+	+	+	−	−	−
Aeromonas hydrophila IAM 1018	−	−	−	−	−	−
A. punctata IAM 1646	−	−	−	−	−	−
A. liquefaciens EFDL	−	−	−	−	−	−
A. salmonicida NCMB 833	−	−	−	+	−	−
A. salmonicida NCMB 1102	−	−	−	+	−	−
A. salmonicida ATCC 14174	−	−	−	+	−	−
A. salmonicida subsp. *masoucida* NCMB 2020	−	−	−	+	−	−
Vibrio metschnikovii IAM 1039	−	−	−	−	−	−
V. tyrogenes IAM 1080	−	−	−	−	−	−
V. piscium var. *japonicus* TUF	−	−	−	−	−	−
V. anguillarum NCMB 6	−	−	−	−	+	−
V. anguillarum NCMB 828	−	−	−	−	+	−
V. anguillarum NCMB 829	−	−	−	−	+	−
V. anguillarum KAY-2	−	−	−	−	+	−
Escherichia coli O-6	−	−	−	−	−	−
Pseudomonas fluorescens EFDL	−	−	−	−	−	−
Micrococcus lysodeikticus Mi 2	−	−	−	−	−	−
Bacillus subtilis NRRL 558	−	−	−	−	−	−

*1 Heat extracted antigen by PBS at 100°C 30 min.
*2 Antiserum provided by National Fish Health Research Lab., Leetown W. Va., U.S.A.
*3 Antiserum against strain Marimo.
*4 Antiserum against strain Otobe.
*5 Normal rabbit serum.
*6 + indicates a positive agglutination reaction; − indicates a negative reaction.

Staphylococci sensitized with unadsorbed antisera showed positive reactions with all antigens employed, but staphylococci sensitized with antisera adsorbed 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 4).

The specificity of the reaction was confirmed with a blocking test. 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 occurred only when the anti-IPNV sensitized staphylococci and unblocked IPNV antigen were used.

By solid phase immune electron microscopy (SPIEM), the agglutinated staphylococci sensitized with anti-IPNV serum would bind IPNV on the

Table 4. Cross coagglutination tests using staphylococci sensitized with IPNV (Buhl), IHNV (Yurappu) 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 Unadsorbed antiserum.
*3 Antiserum adsorbed with FBS and acetone powdered RTG-2 cell.

surface of the cells (Fig. 4).

Coagglutination Test for Rapid Serological Identification of Auto-agglutinating A. salmonicida

Nine *A. salmonicida*, three *Aeromonas* species, two strains of *V. anguillarum*, and *P. fluorescens* were prepared and observed for auto-agglutination. The results of the comparison of auto-agglutination of the bacteria used and the specificity of coagglutination tests using staphylococci sensitized with anti-*A. salmonicida* serum are shown in Table 5. Sensitized staphylococci showed a positive reaction with culture broth and heat-treated cell suspension antigens quickly and produced a large agglutination. The heat extracted antigen showed a positive reaction also, in less than 15 min. Heat extracted antigen is suitable for use in the laboratory or field, because in this preparation causative bacteria are killed and antigen does not include the bacterial cells.

Coagglutination Test for Serological Typing of V. anguillarum

For serological typing of *V. anguillarum* antigens, the specificity of the coagglutination test was evaluated using staphylococci sensitized with three different antisera (EZURA, TAJIMA, YOSHIMIZU and KIMURA, 1981); V-6 (J-O-1 type), V-123 (J-O-2 type) and V-125 (J-O-3 type). Staphylococci sensitized

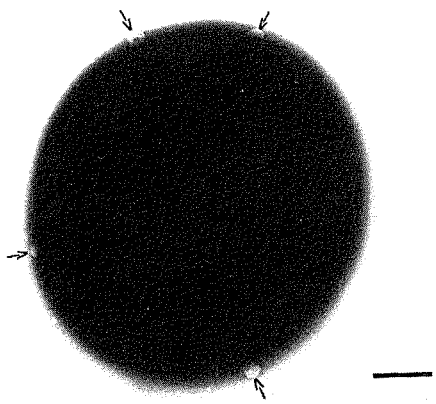

Fig. 4. IPNV adsorbed to *S. aureus* sensitized with homotypic antibody. Arrowheads indicated virus particles. Negatively stained with 0.5% uranyl acetate (pH 5). —— 200 nm.

with anti *V. anguillarum* V-6 rabbit sera showed cross reactions with other strains of the genus *Vibrio*, but staphylococci sensitized with serum adsorbed by these vibrios showed positive reactions only with the strains of *V. anguillarum* belonging to J-O-1. Anti *V. anguillarum* V-123 serum sensitized staphylococci agglutinated with the antigens prepared from the strains V-123, V-114, belonging to the J-O-2 type and anti *V. anguillarum* V-125 serum sensitized staphylococci agglutinated with the anti-

Table 5. Comparison of auto-agglutination of bacteria used and specificity of coagglutination tests using staphylococci sensitized with anti-*A. salmonicida* ATCC 14174 serum

Bacteria or bacterial antigens used	Auto-agglutination	Coagglutination test		
		Culture broth	Heated cell suspension	Heat extract of culture broth
Aeromonas hydrophila IAM 1018	−*1	−	−	−
A. punctata IAM 1646	−	−	−	−
A. liquefaciens EFDL	−	−	−	−
Aeromonas sp.	++*1	−	−	−
A. salmonicida ATCC 14174	−	++	++	+
A. salmonicida NCMB 1102	−	++	++	+
A. salmonicida Nagano N-8	−	++	++	+
A. salmonicida Nagano N-17	+	++	++	+
A. salmonicida Hokkaido 1 M	+	++	++	+
A. salmonicida Hokkaido 5 M	+	++	++	+
A. salmonicida Wakayama	++	++	++	+
A. salmonicida Iwate	++	++	++	+
A. salmonicida subsp. *masoucida* NCMB 2020	−	++	++	+
Vibrio anguillarum NCMB 6	−	−	−	−
Pseudomonas fluorescens EFDL	−	−	−	−

*1 + indicates a positive agglutination reaction; − indicates a negative reaction.

gens prepared from the strains V-125, V-104, V-106, V-117 these are J-O-3 type respectively (Table 6), as reported by NEWMAN, BLOOM and MAJNARICH (1982).

The Coagglutination Test for Serological Typing of Cell Free IPNV Cultured in RTG-2 Cells

For serological typing of cell grown IPNV antigens, three different staphylococci sensitized with antisera against strains VR 299, Sp and Ab were tested against five IPNV strains grown in cell culture from North America, ten strains from Japan and four strains from Europe. 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 only with 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 60 min (Table 7); the agglutinating particles were very fine and different from those in the reactions observed with Sp and Ab strains (Fig. 5). 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 $10^{7.7}$ $TCID_{50}$/m*l*.

The Coagglutination Test with Antibody Sensitized Staphylococci and Kidney Extract Antigen Prepared from Fish with Bacterial Kidney Disease

Heat extracted antigen was prepared from the kidney tissue of a kokanee salmon and a chinook salmon with bacterial kidney disease. The extracts represented a 1 : 5 tissue suspension w/v in PBS. Antigen was similarly prepared from pooled kidney tissue of 17 healthy masu salmon. All three antigens were tested for coagglutination of staphylococci sensitized with antisera specific for the kidney disease bacterium. Two-fold dilutions of the antigens from 2^{-1} to 2^{-6} or higher were employed. Coagglutination reactions occurred with all dilutions of the antigens from the diseased fish. The more concentrated dilutions tended to give positive reactions within 30 min, while the higher dilutions required a longer period. The control antigens prepared from the kidney tissue of healthy fish

Table 6. Specificity of coagglutination tests using staphylococci sensitized with antibody specific for each three serotype *V. anguillarum*

Bacterial antigen used	Anti *V. anguillarum* sera used to sensitize staphylococci									
	Anti V-6 J-O-1		Anti V-6*[1] J-O-1		Anti V-123 J-O-2		Anti V-125 J-O-3		Control	
	a*[2]	b*[3]	a	b	a	b	a	b	a	b
Vibrio anguillarum (V-6) NCMB 6	+*[4]	+	+	+	−	−	−	−	−	−
V. anguillarum (V-123) PTe-1	−*[4]	−	−	−	+	+	−	−	−	−
V. anguillarum (V-125) PT-223	−	−	−	−	−	−	+	+	−	−
Vibrio anguillarum (V-8) NCMB 828	+	+	+	+	−	−	−	−	−	−
V. anguillarum (V-9) NCMB 829	+	+	+	+	−	−	−	−	−	−
V. anguillarum (V-72) KAY-3	+	+	+	+	−	−	−	−	−	−
V. anguillarum (V-105) NOAA 1669	+	+	+	+	−	−	−	−	−	−
V. anguillarum (V-113) PT-24	+	+	+	+	−	−	−	−	−	−
V. anguillarum (V-119) NP-1	+	+	+	+	−	−	−	−	−	−
V. anguillarum (V-114) PT-514	−	−	−	−	+	+	−	−	−	−
V. anguillarum (V-104) NOAA 775	−	−	−	−	−	−	+	+	−	−
V. anguillarum (V-106) N-1	−	−	−	−	−	−	+	+	−	−
V. anguillarum (V-117) NCMB 571	−	−	−	−	−	−	+	+	−	−
V. metschnikovii (V-1) IAM 1039	−	−	−	−	−	−	−	−	−	−
V. tyrogenes (V-2) IAM 1080	−	−	−	−	−	−	−	−	−	−
V. piscium (V-5) TUF	−	−	−	−	−	−	−	−	−	−
V. parahaemolyticus H-O-5	−	±	−	−	−	−	−	−	−	−
V. fischeri NCMB 1281	−	±	−	−	−	−	−	−	−	−
Vibrio sp. A-4-1	±	±	−	−	−	−	−	−	−	−
Lucibacterium harveyi NCMB 1280	±	±	−	−	−	−	−	−	−	−
Aeromonas proteolytica NCMB 1326	−	±	−	−	−	−	−	−	−	−
Beneckea campbelli ATCC 25920	±	+	−	−	−	−	−	−	−	−

*[1] Adsorbed serum using *V. parahaemolyticus*, *V. fischeri*, *V. sp* (*A-4-1*), *L. harveyi*, *A. proteolytica* and *B. campbelli*.

*[2] Heat extracted antigen by PBS at 100°C for 30 min.

*[3] Heated cell suspension antigen; heated in PBS at 100°C for 30 min, after centrifuging the pellet resuspended in PBS.

*[4] + indicates a positive agglutination reaction, − indicates a negative reaction.

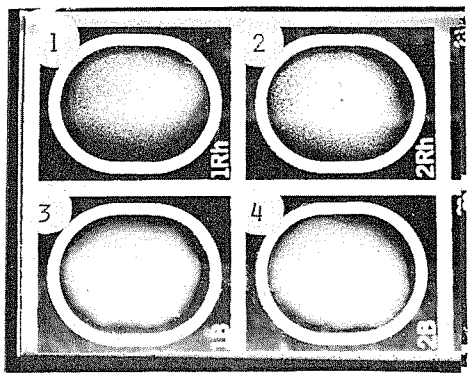

Fig. 5. Coagglutination reaction patterns obtained with MEM_{10} (3), cell free IPNV strains VR-299 (4), Sp (1) and Ab (2), using a staphylococcal suspension sensitized with anti-IPNV (Strain Sp) rabbit serum.

failed to agglutinate the sensitized staphylococci at any concentration tested, and staphylococci sensitized with normal rabbit serum also failed to react with any of the antigen preparations (Table 8).

Data from these experiments indicated the ability of the coagglutination test to detect the specific antigen of bacterial kidney disease in the kidney tissues from infected kokanee and chinook salmon at relatively high dilutions and within a test period of only thirty minutes.

Comparison of the Coagglutination Test with the Classical Diagnostic Methods for the Detection of Bacterial Kidney Disease in Salmonids

The commonly used or classical methods for

Table 7. 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 ($TCID_{50}/ml$)
		Anti-IPNV serum									Control*1			
IPNV	Titer ($TCID_{50}/ml$)	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
Powder 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 (minutes).

Table 8. Effects of concentration of antigen and the time of reaction on the coagglutination tests for detection of BKD antigen in heat extract of affected salmon kidney

Fish specimens	Source of specimens (hatchery)	Concentration of antigen	Antisera used to sensitize staphylococci								
			Anti-BKD bacterium						Control*3		
			Strain EFDL-2*1 Reaction time (min)			Strain AKD-3*2 Reaction time (min)			Reaction time (min)		
			30	60	120	30	60	120	30	60	120
Kokanee*4 salmon No. 19	Chitose	2^{-1}	+*6	+	+	+	+	+	−*6	−	−
		2^{-2}	+	+	+	+	+	+	−	−	−
		2^{-3}	+	+	+	+	+	+	−	−	−
		2^{-4}	+	+	+	+	+	+	−	−	−
		2^{-5}	+	+	+	+	+	+	−	−	−
		2^{-6}	−	−	+	−	−	+	−	−	−
Kokanee*4 salmon No. 29	Erimo	2^{-1}	+	+	+	+	+	+	−	−	−
		2^{-2}	+	+	+	+	+	+	−	−	−
		2^{-3}	+	+	+	+	+	+	−	−	−
		2^{-4}	+	+	+	+	+	+	−	−	−
		2^{-5}	+	+	+	−	+	+	−	−	−
		2^{-6}	+	+	+	−	+	+	−	−	−
		2^{-7}	−	+	+	−	+	+	−	−	−
		2^{-8}	−	−	+	−	−	+	−	−	−
Masu salmon No. 759–776	Mori (Control)	2^{-0}	−	−	−	−	−	−	−	−	−
		2^{-1}	−	−	−	−	−	−	−	−	−
PBS		2^{-0}	−	−	−	−	−	−	−	−	−

*1 Antiserum provided by National Fish Health Research Lab., Leetown W. Va., U.S.A.
*2 Antiserum against strain Marimo.
*3 Normal rabbit serum.
*4 *Oncorhynchus nerka.*
*5 *Oncorhynchus masou.*
*6 + indicates a positive agglutination reaction; − indicates a negative reaction.

diagnosis of bacterial kidney disease include observation of clinical signs, Gram staining of kidney smears, the immunodiffusion test and fluorescent antibody tests. Individual fish, negative by coagglutination, immunodiffusion and the Gram reaction and exhibiting no clinical signs of the disease, were found to be positive by the fluorescent antibody test. Detection of infection by FAT was the most sensitive with coagglutination tests being the next (Table 9). This indicated the effectiveness of FAT for detecting the kidney disease bacterium at very low cell concentrations. However, the increased sensitivity, as compared to immunodiffusion, and lack of a requirement for special U.V. equipment point to the usefulness of coagglutination, especially under field or hatchery conditions (FRYER and SANDERS, 1981).

Diagnosis of Bacterial Kidney Disease by the Coagglutination Test in Fish Populations Undergoing Natural Epizootics of the Disease

A total of six hundred and seventy-four fish specimens were collected at random from nineteen salmonid fish farms thought to be experiencing epizootics of bacterial kidney disease. The number of fish specimens found to be infected with bacterial kidney disease by the coagglutination test were at least as great in all groups of fish examined as the numbers detected by either Gram staining or observation of clinical signs (Table 10).

Table 9. Comparison of clinical signs, gram stain, coagglutination test, direct fluorescent antibody test and immunodiffusion for the detection of bacterial kidney disease or the causative agent in chinook salmon (FRYER and SANDERS, 1981)

Fish number*1	Clinical signs	Gram stain	Coagglutination test*2,4	Fluorescent antibody test*3	Immunodiffusion*4
1	−	+	+	++	+
2	−	+	+	++	+
3	−	−	+	+	−
4	−	−	±	+	−
5	−	+	+	++	+
6	−	+	+	++	+
7	−	−	−	+	−
8	−	−	+	+	−
9	−	−	−	+	−
10	±	+	+	+	+
11	+	+	+	++	+
12	+	+	+	++	+
13	±	+	+	++	+
14	±	+	+	++	+
15	+	+	+	++	+
16	+	+	+	++	+
17	+	+	+	++	+
18	±	+	+	++	+
19	−	+	+	++	+
20	±	+	+	++	+
21	±	+	+	++	+
22	+	+	+	+	+
23	+	+	+	++	+
24	+	+	+	++	+
25	+	+	+	++	+
26	±	+	+	++	+
27	±	+	+	++	+
28	±	+	+	++	+
29	+	+	+	++	+
30	+	+	+	++	+
31	+	+	+	++	+
32	+	+	+	++	−
33	+	+	+	++	−
33 fish	14/33	28/33	30/33	33/33	26/33

*1 Fish 1–30 juvenile; 31–33 adult.
*2 Results after 2 hours incubation at room temperature in a moist chamber.
*3 Smears prepared by heat fixation.
*4 Kidney diluted approximately 1:10 with PBS. Except fish 8–11 and 18, 1:20 and 14, 1:50.

Diagnosis of Furunculosis by the Coagglutination Test

Fish from a total of 70 natural outbreaks of furunculosis and 10 artificially infected fish specimens were examined. The results of comparisons of coagglutination tests, clinical signs and isolation of *A. salmonicida* for diagnosis of furunculosis are shown in Table 11. The number of fish specimens found to be infected with *A. salmonicida* by isolation were greater than by coagglutination tests, but in the case of heat-extracted antigen prepared from furuncle tissue of artificially infected fish, the rate was the same.

Diagnosis of Vibriosis by Coagglutination Test

The comparison of coagglutination tests, clinical

Table 10. Diagnosis of bacterial kidney disease by the coagglutination test in natural outbreaks in Japan, 1977–1980

Fish specimens	Source of specimens (name of farm)	Date	Number of specimens	Number of specimens diagnosed as positive for BKD		
				Clinical sign	Gram stain	Coagglutination test*1
Coho salmon*3	Fujinomiya	Aug. '77	20	13	18	18
Coho salmon	Nakagawa	Aug. '77	7	—*2	—	6
Coho salmon	Koide	Dec. '77	5	4	4	4
Coho salmon	Maebashi	Oct. '78	2	2	2	2
Rainbow trout*4	Fuji	Jap. '79	10	—	—	7
Amago*5	Fuji	May '79	21	2	2	2
Coho salmon	Koide	June '79	5	4	4	4
Masu salmon*6	Otobe	June '79	22	11	12	12
Coho salmon	Miyako-M	June '79	36	12	12	12
Coho salmon	Otuti	July '79	5	1	—	1
Coho salmon	Hamanaka	Aug. '79	15	4	4	6
Masu salmon	Otobe	Sep. '79	10	7	7	8
Coho salmon	Koide	Oct. '79	153	21	65	76
Coho salmon	Fujinomiya	Oct. '79	66	5	9	16
Masu salmon	Otobe	Dec. '79	38	9	18	35
Coho salmon	Kaida	Dec. '79	3	2	2	3
Coho salmon	Otuti	May '80	5	2	—	2
Masu salmon	Koide	May '80	5	4	4	5
Coho salmon	Kukizaki	June '80	20	0	0	3
Masu salmon	Mori	June '80	22	11	—	18
Masu salmon	Koide	June '80	43	35	32	43
Masu salmon	Urasa	June '80	24	10	6	8
Kokanee salmon*7	Shikishima	June '80	10	4	3	9
Coho salmon	Hamanaka	July '80	26	2	0	5
Coho salmon	Otuti	July '80	5	2	—	3
Coho salmon	Toyama	July '80	2	1	1	1
Coho salmon	Hamanaka	Aug. '80	15	13	13	15
Masu salmon	Otobe	Sep. '80	7	3	—	4
Coho salmon	Miyako	Oct. '80	5	2	5	5
Chum salmon*8	Miyako	Oct. '80	47	24	26	34
Coho salmon	Nikko	Oct. '80	3	—	—	2
Masu salmon	Shakotan	Nov. '80	6	2	2	2
Coho salmon	Nikko	Dec. '80	11	2	2	5

*1 Anti-BKD sera; AKD-3, OKD-3, EFDL-1, or EFDL-2; used to sensitize staphylococci.
*2 Indicates no data.
*3 *Oncorhynchus kisutch.*
*4 *Salmo gairdneri.*
*5 *Oncorhynchus rhodurus* var. *macrostomus.*
*6 *O. masou.*
*7 *O. nerka.*
*8 *O. keta.*

signs, and isolation of *V. anguillarum* for the detection of vibriosis was carried out using fish artificially infected with different serotypes of *V. anguillarum*. The number of fish specimens found to be infected with *V. anguillarum* by isolation and coagglutination were the same and the specificity between serotypes was clearly recognized (Table 12).

Table 13 shows the results of detection of *V.*

Table 11. Comparison of coagglutination test, clinical signs, and isolation of *A. salmonicida* for diagnosis of furunculosis

Fish specimens	Source of specimens	Date	Number of fish	Number of specimens with positive diagnosis				
				Clinical sign	Isolation of *A. salmonicida*		Coagglutination test*1	
					Kidney	Furuncle	Kidney*2	Furuncle*2
Coho salmon*3	Iwate	Nov. '79	28	23	19	—*8	15	—
Amago salmon*4	Gifu	Dec. '79	12	6	11	—	6	—
Masu salmon*5	Tokyo	Dec. '79	30	17	28	—	21	—
Rainbow trout*6	Laboratory*7	Mar. '80	10	10	10	10	8	10

*1 Staphylococci sensitized with antiserum against *A. salmonicida* ATCC 14174.
*2 Heat extracted antigen by PBS (1:9) at 100°C for 30 min.
*3 *Oncorhynchus kisutch.*
*4 *Oncorhynchus rhodurus.*
*5 *Oncorhynchus masou.*
*6 *Salmo gairdneri.*
*7 Artificially infected by intramuscular injection.
*8 — indicates no data.

anguillarum antigens by coagglutination testing for natural outbreaks of vibriosis. Except for one case of pen culture coho salmon, all specimens were found to have vibriosis caused by *V. anguillarum* serotype J-O-1. FAT was more sensitive than coagglutination testing, but in some cases coagglutination tests were more sensitive.

Diagnosis of Goldfish Ulcer Disease by Coagglutination Test

Specificity of the coagglutination reaction for detection of atypical *A. salmonicida* antigens was carried out by a cross coagglutination test using staphylococci sensitized with anti-atypical *A. salmonicida* and anti-typical *A. salmonicida* sera. Atypical *A. salmonicida* and typical *A. salmonicida* have one common antigen (KIMURA and YOSHIMIZU, 1984b). These three sensitized staphylococci all showed positive reactions with typical *A. salmonicida* and atypical *A. salmonicida* strains (Table 14).

Results of detection of an atypical *A. salmonicida* in affected tissues and kidneys of goldfish are shown in Table 15. All specimens prepared from diseased fish showed positive agglutination. However, except for one specimen, we failed to isolate the atypical *A. salmonicida*. Agglutination antibody titers of diseased fish were relatively high.

Diagnosis of IPN in Fish Undergoing Natural Epizootics using the Coagglutination Test

A total of 44 fish specimens consisting of three salmonid species were collected at random from six fish farms. Three healthy chum salmon fry were used for controls. Staphylococci sensitized with antiserum against IPNV (strain Buhl) showed positive coagglutination reactions with tissue from rainbow trout, coho and amago salmon cultured in Gifu Prefecture. The virus was isolated from all fish except the coho salmon Nos. Co-4 to 6 which were received by our laboratory chilled with dry ice. The antigens prepared from the rainbow trout cultured in Yamanashi and Niigata Prefectures, from which IPNV was not isolated, and from rainbow trout cultured in Niigata, from which IHNV was isolated, showed negative reactions. The control antigens prepared from healthy chum salmon also showed negative reactions (Table 16).

Conclusions

The application of the coagglutination test using staphylococci specifically sensitized with antibodies against the bacteria causing BKD, furunculosis, vibriosis and goldfish ulcer disease, and also against the virus causing IPN, for the diagnosis of these

Table 12. Comparison of coagglutination test, clinical signs, and isolation of *V. anguillarum* for detection of vibriosis

Examination	Fish specimens	Injection strain	Serotype of strain	Number of fish	Number of specimens with positive diagnoses										
					Clinical sign	Isolation of *V. anguillarum*		Coagglutination test; sensitized with							
								Anti V-6		Anti V-123		Anti V-125		Control	
						Kidney	Liver	K*1	L*2	K	L	K	L	K	L
I*3 (July '80)	chum salmon	V-6	J-O-1	11	10	7	7	7	4	0	0	0	0	0	0
	chum salmon	V-123	J-O-2	10	5	5	5	0	0	5	5	0	0	0	0
	chum salmon	V-106	J-O-3	34	28	7	7	0	0	0	0	7	7	0	0
II*4 (Jan. '81)	chum salmon	V-6	J-O-1	10	8	8	—	8	—	0	—	0	—	0	—
	chum salmon	V-123	J-O-2	10	1	1	—	0	—	1	—	0	—	0	—
	chum salmon	V-125	J-O-3	10	10	10	—	0	—	0	—	10	—	0	—

*1 Kidney.
*2 Liver.
*3 Intracavity injection; V-6, 2.0×10^7; V-123, 4.8×10^6; V-106, 8.0×10^6.
*4 Intramuscular injection; V-6, 1.8×10^6; V-123, 2.0×10^6; V-125, 4.7×10^6.

Table 13. Diagnosis of vibriosis by the coagglutination test and FA method in natural outbreaks

Source of specimens	Fish species	Date	Number of specimens	Number of specimens diagnosed as positive for vibriosis					
				Clinical sign	Coagglutination test; sensitized with				FA method; indirect by anti V-6 serum
					Anti V-6	Anti V-123	Anti V-125	Control	
Yamanashi-B	rainbow trout*2	July '81	10	—	6	0	0	0	4
Yamanashi-B	rainbow trout	July '81	10	—	5	0	0	0	2
Tochigi-S	ayu*1	July '81	6	0	5	0	0	0	6
Tochigi-O	ayu	July '81	8	3	8	0	0	0	8
Tochigi-O	ayu	Aug. '81	4	0	2	0	0	0	4
Tochigi-I	ayu	Aug. '81	15	8	9	0	0	0	11
Miyagi-S*5	coho salmon*3	Dec. '81	3	—	0	1	2	0	—
Hokkaido-M*6	chum salmon*4	Dec. '81	9	3	5	0	0	0	8

*1 *Plecoglossus altivelis.*
*2 *Salmo gairdneri.*
*3 *Oncorhynchus kisutch.*
*4 Sea water fish, serotype of isolates was J-O-3.
*5 *O. keta.*
*6 Cultured in aquarium by sea water, serotype of isolates was J-O-1.

Table 14. Specificity of coagglutination test using staphylococci sensitized with anti *A. salmonicida* and atypical *A. salmonicida*

Bacterial antigen used*	Antisera used to sensitize staphylococci		
	Anti-*A. salmonicida*	Anti-atypical *A. salmonicida*	
	ATCC 14174	V-76-65	V-76-134
Atypical *A. salmonicida*			
LO-2	+	+	+
LO-6	+	+	+
Atypical *A. salmonicida*			
TY-76192	+	+	+
TY-79057h	+	+	+
TY-79058f	+	+	+
TY-790591	+	+	+
TY-80003w	+	+	+
Atypical *A. salmonicida*			
V-76-65	+	+	+
V-76-134	+	+	+
A. salmonicida ATCC 14174	+	+	+
NCMB 1102	+	+	+
A. salmonicida subsp. *masoucida* NCMB 2020	+	+	+
A. hydrophila NCMB 86	–	–	–
A. punctata NCMB 74	–	–	–
A. liquefaciens ATCC 11715	–	–	–

* Heat extracted antigen by PBS at 100°C 30 min.

diseases in fish was studied.

Stabilized *Staphylococcus aureus* (COWAN I) was sensitized with rabbit anti-serum against *R. salmoninarum*, *V. anguillarum*, typical and atypical *A. salmonicida* and IPNV.

The antibody binding ability of the sensitized staphylococci was tested and the agglutinating antibody titers of antisera were markedly reduced after incubation with the stabilized staphylococci.

Specificity of the coagglutination reaction for detection of bacterial and viral antigens in vitro were carried out by a cross coagglutination test. Agglutination occurred only with specific combinations of antibody sensitized staphylococci and the homologous antigen for the heat extracted bacteria, the heated bacteria and the non-treated cell free viral antigens, and not with heterologous antigens or with staphylococci sensitized with normal rabbit serum. By SPIEM, the agglutinated staphylococci sensitized with anti-IPNV serum would bind IPNV on the surface of the cells.

In accordance with this high specificity, the coagglutination test is useful for rapid serological identification of auto-agglutinating *A. salmonicida*, as well as for serological typing of *V. anguillarum* using the antisera against three O-antigens (J-O-1 to J-O-3) of *V. anguillarum*. Also it is useful for serological typing of IPNV of at least two groups, the North American and European strains.

The most important use of the coagglutination test in the diagnosis of fish disease would be to detect the specific bacterial and viral antigens in the tissue of infected fish. It was found that bacterial antigens could be detected in heat extracts of kidney, furuncles or affected tissues and that viral antigen could be detected in extracts of whole

Table 15. Diagnosis of ulcerative furunculosis by the coagglutination test

Fish No.	Pond No.	Species	Clinical sign		Isolation of of atypical *A. salmonicida*	Coagglutination test*1		Agglutinating antibody titer*3
						Kidney*2	Affected*2 tissue	
1	A	Gold fish	+	early	−	+	+	1:128
2	〃	〃	+	〃	−	+	+	1:128
3	〃	〃	+	〃	−	+	+	1:128
4	〃	〃	+	〃	−	+	+	1:512
5	〃	〃	+	〃	−	+	+	1:128
6	〃	〃	+	〃	−	+	+	1:256
7	〃	〃	+	〃	−	+	+	1:512
8	〃	〃	+	〃	−	+	+	1:256
9	〃	〃	+	〃	−	+	+	1:256
10	〃	〃	+	〃	−	+	+	1:128
11	B	Gold fish	+	early	−	+	+	1:128
12	〃	〃	+	〃	−	+	+	1: 8
13	〃	〃	+	〃	−	−	+	1: 8
14	C	Gold fish	+	early	+	+	+	1:128
15	〃	〃	+	〃	−	+	+	1: 8
16	〃	〃	+	〃	−	−	+	1: 8
17	D	Crucian carp	−	normal	−	−	−	ND*4
18	〃	〃	−	〃	−	−	−	ND
19	〃	〃	−	〃	−	−	−	ND
20	〃	〃	−	〃	−	−	−	ND
21	〃	〃	−	〃	−	−	−	ND

*1 Anti *A. salmonicida* ATCC 14174 rabbit serum sensitized staphylococci.
*2 Heat extracted antigen.
*3 Heated cell suspension of atypical *A. salmonicida* V-76-134 was used for antigen.
*4 Not determined.

viscera of infected fish at relatively high dilutions (5 or 10×2^{-1} to 2^{-6}) and within a test period of 30 min.

This method proved to be a simple, rapid and reliable diagnostic test suitable for use in the laboratory or field, and one which required no special apparatus.

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Table 16. 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-3 to R-16	Rainbow trout	Yamanashi	−	ND	−	ND
R-17 to R-25	Rainbow trout	Yamanashi	−	ND	−	ND
R-26 to R-30	Rainbow trout	Niigata	+ (INNV)*5	3.05	−	ND
R-31 to R-35	Rainbow trout	Niigata	+ (IHNV)*5	1.05	−	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.
*5 IHNV was isolated from one fish.

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