

Asian Fisheries Science 4(1991): 23-30.
Asian Fisheries Society, Manila, Philippines
<https://doi.org/10.33997/j.afs.1991.4.1.003>

Diagnostic Application of Monoclonal Antibodies Against the Heat Extract Antigen of *Renibacterium salmoninarum*, the Causative Agent of Bacterial Kidney Disease

MASAHIRO SAKAI
SHIZUO ATSUTA
MASANORI KOBAYASHI

*School of Fisheries Sciences
Kitasato University
Sanriku, Iwate, 022-01
Japan*

Abstract

Two monoclonal antibodies (JK-1 and JK-2) were made against the heat extract antigen of *Renibacterium salmoninarum*. These monoclonal antibodies recognized a 60 kd and several lower molecular weight protein antigens extracted by heating. The subclass of monoclonal antibody was IgG 2 in both clones, and did not react with six other bacterial fish pathogens in the dot blot assay. Using the dot blot assay, these monoclonal antibodies and a polyclonal antibody were used to detect *R. salmoninarum* antigen from coho salmon, *Oncorhynchus kisutch*, and both clones showed equal sensitivities using the polyclonal antibody.

Introduction

Bacterial kidney disease (BKD) is one of the most serious salmonid diseases in many countries. *Renibacterium salmoninarum*, the causative agent of this disease, is a Gram-positive diplobacillus and this bacterium requires a long incubation time before its colonies appear. The disease is not prevented by the administration of chemotherapeutants or vaccination (Elliott et al. 1989), thus control is very difficult.

Many immunological procedures to detect the *R. salmoninarum* antigen from diseased fish have been reported: an immunodiffusion method (Chen et al. 1974); fluorescent antibody technique (FAT)

(Bullock and Stuckey 1975); coagglutination test (Kimura and Yoshimizu 1981); dot blot assay (Sakai et al. 1987); and enzyme-linked immunosorbent assay (ELISA) (Pascho and Mulcahy 1987). These methods use a polyclonal antibody against *R. salmoninarum*, and it is possible that this antibody could also react with other bacteria non-specifically. In fact, Austin et al. (1985) and Yoshimizu et al. (1987) reported the existence of bacteria reacting with *R. salmoninarum* polyclonal antibody. Truga et al. (1987) introduced the western blot system, gave detailed information on the antigens and discussed the diagnostic possibility of this method to BKD. However, this method is expensive and time consuming.

Recently, the monoclonal antibody technique has been applied to the diagnosis of disease. Arakawa et al. (1987) developed monoclonal antibodies to *R. salmoninarum* cells, but these antibodies reacted with other bacteria. In this study, two monoclonal antibodies to *R. salmoninarum* heat extract antigen were established and were applied to diagnosis of BKD.

Materials and Methods

Bacteria Strains

R. salmoninarum KU8501 isolated from moribund coho salmon, *Oncorhynchus kisutch*, in 1985 at Iwate, Japan, was used in this study. Bacteria was grown on KDM-2 (Evelyn 1971) and incubated for 14 days at 15°C.

Antigen Preparation

R. salmoninarum cells were suspended in 0.85% saline (PS) and inactivated by adding 0.3% formalin. After washing three times with PS, the concentration was adjusted to an optical density (OD) of 1.0 at 545 nm. Equal volumes of cell suspension and Freund's complete adjuvant (FCA) (Difco) were mixed and this mixture was used as the antigen for monoclonal or polyclonal antibody preparations.

Production of Monoclonal Antibodies

BALB/cx mice were immunized with 0.1 ml of the cell suspension in FCA and boosted with 0.05 ml of antigen without adjuvant after two and three weeks. Monoclonal antibody-secreting cells were produced by a modification of the protocol used by Kohler and Milstein (1975). Mouse spleen cells were fused with P3/X63-AG8 myeloma cells. Cultured medium was collected for the ELISA. The hybridomas demonstrating *R. salmoninarum* antibody production were cloned twice by limiting dilution. Two of these clones were chosen for ascites production. Approximately 1×10^6 cells from these hybridomas were injected into BALB/c mice that had been given 0.5 ml of pristine seven days previously. Ascites fluid was collected 10 days later and clarified by centrifugation.

Preparation of Polyclonal Antibodies

One ml of the antigen mixture was inoculated intramuscularly into each leg of a rabbit. After two weeks, a booster immunization was given intravenously using 1 ml of the cell suspension without adjuvant. The rabbit was exsanguinated 10 days after the second injection.

ELISA

R. salmoninarum cells, adjusted to the concentration of OD 1.0, were heated for 10 min at 100°C and centrifuged. Supernatants were used as the antigen to select hybridomas. The antigen solution was diluted with 0.1 M carbonate-bicarbonate buffer (pH 9.6). Individual wells of a 96-well EIA flat-bottom plate were coated with 50 μ l of the antigen solution and incubated overnight at 4°C. Following five rinses of the plates with 0.05% Tween 80 in Tris-buffer saline, pH 8.2 (TTBS), and two rinses of the plates with distilled water (DW), the wells were blocked by a 30 min incubation with 3% bovine serum albumin (BSA) in TBS. After washing as described above, 100 μ l of each hybridoma supernatant was incubated for 2 hours at 37°C. The plates were then washed and 50 μ l of horseradish peroxidase-conjugated antimouse-Ig goat IgG were incubated in each well for 30 min at 37°C. After the last rinse, 100 μ l of substrate solution (10 μ l of citrate buffer (pH 4.0), 10 μ l of hydrogen peroxide and 75 μ l of a 10

mg/ml solution of 2, 2'-Azinobis, 3-ethyl benzthiazoline sulfonic acid (ABTS in DW) were added and absorbance was measured at 405 nm on an EIA reader (Bio Rad).

Polyacrylamide Gel Electrophoresis and Western Blotting

Ten μ l of the heat-extract antigen of *R. salmoninarum* and 30 μ l of sample buffer consisting of 0.5 M Tris-HCl, 10% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.5% bromophenol blue were mixed and heated for 5 min at 100°C. Ten μ l of the samples underwent electrophoresis in a 15% acrylamide separating gel and a 5% acrylamide stacking gel and run for 4 hours with a constant current of 20 mA. The proteins were transferred from the gel to nitrocellulose membrane by electrophoresis at 0.5 mA for 90 min at 4°C (Advantic). This membrane was blocked for 15 min at 37°C with 3% BSA diluted in TBS. Anti-*R. salmoninarum* monoclonal or polyclonal antibodies were allowed to react in this membrane for 1 hour at 20°C and washed five times with TTBS. Peroxidase-conjugated antimouse-Ig or antirabbit-Ig goat Ig was applied to the blot for 1 hour at 20°C after which it was rinsed by the same method. Visualization of the reactive protein band was achieved using DAB (Sakai et al. 1989). *R. salmoninarum* monoclonal antibodies 4D3 and 2G5 established by Wiens and Kaattari (1989) were used as controls.

Isotype Analysis

Ouchterlony immunodiffusion (1962) was performed to determine the Ig class and subclass of the monoclonal antibodies. Undiluted monoclonal antibodies were tested against goat antimouse IgG, IgG1, IgG2a, IgG2b, IgG3 and IgM sera (producer) using a 1% agarose gel.

Specificity of Monoclonal Antibodies

Specificity was determined by testing cross-reactivity with other fish pathogens including: *Aeromonas salmonicida* ATCC14174; *A. hydrophilia* KAH8501; *Pseudomonas fluorescens* 8401; *Edwardsiella tarda* AJC8101; *Vibrio anguillarum* PT24; *V. ordarii* KV08501; and

Streptococcus sp. SG8101. All bacteria were cultured on Brain Heart Infusion (BHI) (Nissui) for 48 hours at 20-30°C. Heat extract antigens were prepared from these bacteria and specificity was examined with the indirect dot blot assay (Sakai et al. 1989).

Application for Detection of Naturally Occurring BKD

O. kisutch, naturally infected with BKD, were examined. We collected 149 fish (about 150 g each) from six freshwater fish farms in Iwate Prefecture. Fish were examined for clinical signs and the dot blot assay was performed using monoclonal antibodies or polyclonal antibody.

Results

Two monoclonal antibodies (JK-1 and JK-2) were established. These antibodies reacted negatively with all other examined bacterial heat extract antigens (Table 1). Ouchterlony analysis of ascites fluids from each clone against subclass-specific antisera indicated that both clones were IgG2b.

Both monoclonal antibodies reacted with one major protein (MW about 60 kd) and several minor proteins (MW about 40 in the heat extract antigen of *R. salmoninarum*). These antibodies recognized the same proteins with which monoclonal antibodies 4D3 and 2B5 reacted.

Table 1. Specificities of *Renibacterium salmoninarum* monoclonal antibody JK-1 and JK-2.

Bacterial antigen	JK-1	JK-2
<i>Renibacterium salmoninarum</i> KU8501	+	+
<i>Renibacterium salmoninarum</i> KU8502	+	+
<i>Renibacterium salmoninarum</i> KU8504	+	+
<i>Renibacterium salmoninarum</i> RB173	+	+
<i>Aeromonas salmonicida</i> ATCC14174	-	-
<i>Aeromonas hydrophila</i> KAH8501	-	-
<i>Pseudomonas fluorescens</i> 8401	-	-
<i>Edwardsiella tarda</i> AJC8101	-	-
<i>Vibrio anguillarum</i> PT24	-	-
<i>Vibrio ordalii</i> KV08501	-	-
<i>Streptococcus</i> sp. SG8101	-	-

One of the salmon from the fish farms had developed clinical signs of BKD (abscess of the kidney). In the dot blot assay, the polyclonal antibody showed 32 samples to be positive. Monoclonal antibodies JK-1 and JK-2 detected 27 and 28 positive samples, respectively (Table 2).

Table 2. Detection of *Renibacterium salmoninarum* antigen, using the monoclonal antibody JK-1, JK-2 and polyclonal antibody. The assay was examined by the indirect dot blot method.

Farm	Number of specimens examined	Polyclonal antibody	Monoclonal antibody	
			JK-1	JK-2
A	39	10	7	8
B	20	7	4	4
C	20	3	6	5
D	20	1	2	1
E	32	6	4	3
F	18	5	4	5
Total	149	32	27	26

Discussion

R. salmoninarum monoclonal antibodies have already been reported by Arakawa et al. (1987) and Wiens and Kaattari (1989). Arakawa et al. (1987) have produced monoclonal antibodies to a heat stable antigen, but this antibody was shown to cross-react in ELISA with three other species of Gram-positive bacteria. The antibodies reported here did not show any cross-reactivity against the investigated Gram-negative bacterial strains using the dot blot assay. Austin et al. (1985) and Yoshimizu et al. (1987) reported that bacteria cross-reacted with *R. salmoninarum* polyclonal antibody in the IFAT. We did not examine the cross-reactivity with these bacteria in this study, but further work is needed to test the cross-reactivity between our monoclonal antibodies and these bacterial strains.

Wiens and Kaattari (1989) established two monoclonal antibodies which recognized a 57-kd protein doublet and several lower molecular weight antigens of *R. salmoninarum* lysates. Our monoclonal antibodies recognized a 60-kd and some lower molecular weight proteins of heat extract antigen of *R. salmoninarum* cells. Wien's and our monoclonal antibodies recognized the same proteins in western blot experiments. Thus, we suggest that our heat-stable antigen includes the antigen of *R. salmoninarum* lysates.

Our monoclonal antibodies could detect *R. salmoninarum* antigen from BKD-infected fish or carrier fish using dot blot assay; and these sensitivities were almost equal to that of *R. salmoninarum* polyclonal antibody. Sakai et al. (1989) already reported that the dot blot assay is a very sensitive method to detect *R. salmoninarum* antigen, although some false positive reactions were observed. These results indicate that the antibodies reported here are useful for the BKD carrier fish. More field investigation is required to examine the diagnostic values of these antibodies.

Acknowledgements

This study was funded in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan (63760158). The P3/X63-AG8 cell line was supplied by the Japanese Cancer Research Resources Bank. *R. salmoninarum* monoclonal antibodies 4D3 and 2D5 were kindly given by Dr. J. Winton, National Fisheries Service, USA.

References

- Arakawa, C.K., J.E. Sanders and J.L. Fryer. 1987. Production of monoclonal antibodies against *Renibacterium salmoninarum*. *J. Fish Dis.* 10: 249-253.
- Austin, B., D. Bucke, S. Feist and J. Rayment. 1985. A false positive reaction in the indirect fluorescent antibody test for *Renibacterium salmoninarum* with a coryneform organism. *Bull. Eur. Ass. Fish. Path.* 5: 8-9.
- Bullock, G.L. and H.M. Stuckey. 1975. Fluorescent antibody identification and detection of the *Corynebacterium* causing kidney disease of salmonids. *J. Fish. Res. Board Can.* 32: 2224-2227.
- Chen, P.K., G.L. Bullock, H.M. Stuckey and A.C. Bullock. 1974. Serological diagnosis of corynebacterial kidney disease of salmonids. *J. Fish. Res. Board Can.* 31: 1939-1940.
- Elliott, D.G., R.J. Pascho and G.L. Bullock. 1989. Developments in the control of bacterial kidney disease of salmonid fishes. *Dis. Aquat. Org.* 6: 201-215.
- Evelyn, T.P.T. 1971. An improved growth medium for the kidney bacterium and some notes in using the medium. *Bull. Off. Int. Epizo.* 87: 511-513.
- Kimura, T. and M. Yoshimizu. 1981. Rapid method for detection of bacterial kidney disease of salmonid (BKD) by coagglutination of antibody sensitized protein A containing staphylococci. *Bull. Jap. Soc. Sci. Fish.* 47: 1173-1183. (In Japanese with English summary).
- Kohler, G. and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of redefined specificity. *Nature* 256: 495-497.
- Ouchterlony, O. 1962. Diffusion-in-gel methods for immunological analysis II. *Progr. Allergy* 6: 30-154.
- Pascho, R.J. and D. Mulcahy. 1987. Enzyme-linked immunosorbent assay for a soluble antigen of *Renibacterium salmoninarum* the causative agent of salmonid bacterial kidney disease. *Can. J. Fish. Aquat. Sci.* 44: 183-191.

- Sakai, M., S. Atsuta and M. Kobayashi. 1989. Comparison of serodiagnostic methods used to detect bacterial kidney disease. *J. Aqua. Anim. Health* 1: 21-24.
- Truga, P.S.D., G.D. Wiens and S.L. Kaattari. 1987. Analysis of *Renibacterium salmoninarum* antigen production *in situ*. *Fish Path.* 22: 209-214.
- Wiens, G.D. and S.L. Kaattari. 1989. Monoclonal antibody of common surface protein(s) of *Renibacterium salmoninarum*. *Fish Path.* 24: 1-7.
- Yoshimizu, M., R. Ji. T. Nomura and T. Kimura. 1987. A false positive reaction in the indirect fluorescent antibody test for *Renibacterium salmoninarum* ATCC 33209 caused by a *Pseudomonas* sp. *Sci. Rep. Hokkaido Salm. Hatch.* 41: 121-127. (In Japanese with English summary).