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Monoclonal Antibodies against *Aeromonas salmonicida* for Serological Diagnosis of Furunculosis

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Hybridomas which produce species-specific and strain-specific monoclonal antibodies (MAbs) against *Aeromonas salmonicida* have been established. Reaction of three MAbs, Nos. 9, 17, and 40, was strain-specific while one MAb, No. 27, was species-specific. MAb 27 reacted with *A. salmonicida* subsp. *salmonicida*, *achromogenes*, *masoucida*, and atypical *A. salmonicida*. The titer of this MAb, a culture fluid of hybridoma, was 1: 8, 1: 256, 1: 8, and 1: 128 for agglutination test, ELISA, FAT, and colony blotting test, respectively. The molecular weight of the antigen recognized by MAb 27 was 51 to 62 kDa.

Aeromonas salmonicida is the etiologic agent of furunculosis in salmonid fish, erythrodermatitis in carp, and ulcerative disease in goldfish, and has been a world economic threat to the intensive culture of these fish for over fifty years.[1)]

Under ordinary circumstances, in cases of classical furunculosis caused by typical starins of *A. salmonicida*, the pathogen may readily be recovered from diseased fish especially from surface lesions or the kidney using non-selective bacteriological agar media. However, non-pigmented strains of *A. salmonicida*; *A. salmonicida* subsp. *achromogenes* and subsp. *masoucida* have been reported. Also some strains of other bacteria, *A. hydrophila* and *A. media* produce a diffusible brown pigment. In addition, if the fish have succumbed to secondary infection with other microorganisms, isolation of *A. salmonicida* becomes much more difficult because of the overgrowth of other bacteria. Thus, growth of *A. salmonicida* may be suppressed and pigment production is inhibited.[2)]

The presence of common antigens among several species of *Aeromonas* and *Vibrio* have been reported[3),4)] and cross reactions cause problems in sero-diagnosis of furunculosis. Additionally, *A. salmonicida* isolated from diseased fish showed autoagglutination on a standard slide agglutination test; therefor other methods were required[5)] for serological identification of *A. salmonicida*. To establish a rapid simple diagnostic method based on serological techniques, highly species-specfic antisera are necessary. We have succeeded in establishing a hybridoma which produces a highly specific monoclonal antibody against *A. salmonicida*.

Materials and Methds

Antigen Preparation and Immunization of Mice

A. salmonicida subsp. *salmonicida* strain ATCC 14174 was cultured in trypticase soy broth (TSB) at 25°C for 48 h. The culture broth was centrifuged at 3,000×g for 20 min and washed 3 times with phosphate buffered saline (PBS). After washing, the bacterial suspension was heated at 100°C for 30 min and centrifuged at 4,000×g for 20 min. The bacterium was resuspended in PBS (0.1% NaN_3) and used as a heat-killed cell antigen.

Six-week-old female BALB/c mice were immunized subcutaneously every week for 5 times with 0.3 m*l* of antigen. Fusion was performed 4 days after the last immunization.

Preparation of Hybridoma

Myeloma cell (SP2/O-Ag 14), provided by Dr. Kida of the Faculty of Veterinary Medicine, Hokkaido University, was maintained in enriched RDF medium (Daigo) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 I.U./m*l* of penicillin and 100 I.U./m*l* of streptomycin (Sigma) in 5% CO_2 at 37°C.

According to the method of Kida *et al.*,[6)] the spleen cells and myeloma cells were mixed at a

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ratio of 10: 1 and centrifuged. Mixed cells were suspended in small amounts of E-RDF medium, then 50% polyethylene glycol (PEG, M.W. 1500 Sigma) was added to the mixed cells. The fusion mixture was diluted by the medium. The cells were then washed and resuspended in an E-RDF medium containing hypoxanthin aminopterin thymidine (HAT, Sigma) at 3.5×10^6/ml. The fusion mixture was poured into 96 wells at 0.1 ml/well. The plates were then placed in a 5% CO_2 incubator at 37°C.

The hybridoma cells were transferred to 24 well plates for tissue culture (Conring) and incubated at 37°C until the well contained visible growth of the cells. Culture medium of hybridoma was assayed by enzyme-linked immunosorbent assay (ELISA) described below.

ELISA for Screening and Cloning of the Hybridoma

Fifty μl of heat-killed bacterial cell suspension (10^8 cell/ml) was poured into each well of a 96-well plate and fixed at 37°C overnight. The plates were washed 3 times in PBS-Tween, then 50 μl of 2% skimmed milk was added and incubated at 37°C for 1 h. Antibody in the cultured medium of the hybridoma was screened by ELISA according to the method described by Campbell.[7]

The selected hybridomas were diluted in 2-fold dilution and inoculated into 96 well plates with feeder cells. Each clone was expanded by transference into 24 well plates.

Production MAb

Each of the cloned hybridoma was cultured in E-RDF supplemented with 10% Nuserum (Maruzen) until the cell number increased to $6\sim7\times10^8$ cells/ml. The antibody was recovered by centrifugation at $1,200\times g$ for 10 min.

Immunoglobulin Classification of MAb

MAbs were classified by mouse monoclone Typing Kit (The Binding Site LTD.) using antibodies against mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM.

Specificity of MAb

Sixteen strains of *A. salmonicida* subsp. *salmonicida*, 1 strain of *A. salmonicida* subsp. *achromogenes*, 2 strains of *A. salmonicida* subsp. *masoucida*, 3 strains of atypical *A. salmonicida*, 2 strains of *Aeromonas* sp. (*A. pseudosalmonicida*[6]), and 6 strains of other fish pathogens; 4 strains of *A. hydrophila* (including *A. liquefaciens* and *A. punctata*), 2 strains of *V. anguillarum*, and 1 strain of *E. coli* antigens were prepared for ELISA and colony blotting test and specificity of the MAbs was estimated. One strain of *A. salmonicida* subsp. *salmonicida* ATCC 14147, which did not show autoagglutination, was prepared for an agglutination test and FAT.

Titer of MAb

MAb was diluted as in 2 fold dilution series and the maximum dilution that reacted positively in a slide agglutination test, a colony blotting test, ELISA, and FAT was determined.

Sensitivity of MAb

Heat-killed antigen of *A. salmonicida* strain ATCC 14174 (10^8 cells/ml) was diluted by 10-fold dilution. Fifty μl of each dilution was added to 8 wells of a 96-well plafe and tested by ELISA to determine the lowest concentration of the antigen that showed a positive reaction.

Antigen Determination

Seven strains of the bacteria, 3 strains of *A. salmonicida* subsp. *salmonicida* and *A. hydrophila*, and 1 strain of *A. salmonicida* subsp. *masoucida*, were treated with 100 μl of denaturing buffer and heated at 100°C for 3 min. After cooling, 10 μl of BPB buffer (0.5% w/v bromophenol blue, 70% glycerol, 0.0625 M Tris pH 6.8) were added and mixed (kept at −20°C until use). The samples were electrophorized in a 10% SDS-polyacrylamide gel according to the method of Lammli. The gel was stained with coomassie brilliant blue. Then the proteins were transferred from the gel to a nitrocellulose membrane (Millipore). The other nonspecific proteins were blocked by 2% skimmed milk at room temperature overnight. After washing in Tris buffered saline, monoclonal antibody was allowed to react with the blots. Blots were stained by the immuno-peroxidase method.[7]

IFAT

Heat-killed bacteria were smeared on to a glass slide and fixed by heating. Fifty μl of MAb was added to the fixed bacteria and the reaction was carried out in a moist chamber at 37°C for 30 min. Fixed cells were washed 4 times in Tween-PBS and FITC-conjugated swine anti-mouse IgG (1: 100; Dakopatts) was reacted at

37°C for 30 min. The slide glass was again washed 3 times with Tween-PBS, mounted with glycerin buffer pH 9.0, and observed by a fluorescence microscope (Olympus, Vanox).

Agglutination Test

A volume of 50 μl of antibody and 50 μl of heat-killed antigen was mixed on a glass slide and the reaction was carried out at room temperature for a few minutes. The slide was examined with the naked eye or under a microscope ($\times 10$) for the development of agglutination reaction.

Colony Blotting Test

Bacteria employed were spot-cultured on fresh-water agar (FWA) plate[6] and incubated at 25°C. After 24 h incubation, the nitrocellulose membrane (90 mm diameter, Millipore) was placed on the agar plate for about 2 min. Blotted bacteria were killed and fixed by heating at 50°C for 2 h. The membrane was reacted with 0.3% H_2O_2 for 20 min. After washing with TBS 3 times, the membrane was incubated in a blocking solution (2% skimmed milk in TBS) at room temperature overnight to block the remaining protein binding sites. The membrane was stained by immuno-peroxidase methods.[7]

Results

Characterization of Monoclonal Antibody

After cloning 3 times, twenty hybridomas showed positive reaction by FAT and ELISA, and four hybridomas, Nos. 9, 17, 27, and 40, grew well and were selected for a further studies. These culture media including monoclonal antibodies were used to determine the immunoglobulin subclass. Three monoclonal antibodies MAb-9, 17, and 40 were classified to be IgG1 and MAb-27 was classified as IgG2a and IgG3.

Specificity of Monoclonal Antibody

Specificity of 4 MAbs, MAb-9, 17, 27, and 40 were tested by ELISA and FAT using 3 strains of *A. hydrophila*, 4 strains of *A. salmonicida* subsp. *salmonicida*, 1 strain of *A. salmonicida* subsp. *achromogenes* and subsp. *masoucida*, 2 strains of *V. anguillarum*, and 1 strain of *E. coli*. As shown in Table 1, monoclonal antibodies MAb-9, 17, and 40 were strain-specific and reacted only with *A. salmonicida* strain ATCC 14174, while MAb-27 reacted with other strains of *A. salmonicida* including subsp. *achromogenes* and *masoucida*. However, not all the MAbs showed cross reaction with other species of bacteria tested.

Again, specificity was tested using 17 strains of *A. salmonicida* subsp. *salmonicida*, 1 strain of *A. salmonicida* subsp. *achromogenes*, 2 strains of *A. salmonicida* subsp. *masoucida*, 3 strains of atypical *A. salmonicida*, 2 strains of *Aeromonas* sp. (*A. pseudosalmonicida*), 6 strains of other fish pathogenic bacteria, and 1 strain of *E. coli* by a colony blotting test and FAT. MAb-27 showed positive reaction with *A. salmonicida* subsp. *salmonicida*, subsp. *masoucida*, and atypical *A. salmonicida*, but did not show any reaction with other strains of *Aeromonas*, *Vibrio*, or *Escherichia* (Table 2).

Titer of MAb

Maximum dilution of MAb-27 able to detect the *A. salmonicida* ATCC 14174 antigen was determined by an agglutination test, ELISA, FAT,

Table 1. Reaction of 4 MAbs with several fish pathogens by ELISA and FAT

Bacterial antigen used	MAb			
	9	17	27	40
Aeromonas hydrophila (IAM 1018)	−	−	−	−
A. hydrophila (*A. liquefaciens* EFDL)	−	−	−	−
A. hydrophila (*A. punctata* NCMB 74)	−	−	−	−
A. salmonicida subsp. *salmonicida* (ATCC 14174)	+	+	+	+
A. salmonicida subsp. *salmonicida* (NCMB 1122)	−	−	+	−
A. salmonicida subsp. *salmonicida* (Shiga)*	−	−	+	−
A. salmonicida subsp. *salmonicida* (Nagano)*	−	−	+	−
A. salmonicida subsp. *achromogenes* (NCMB 1110)	−	−	+	−
A. salmonicida subsp. *masoucida* (NCMB 2020)	−	−	+	−
Vibrio anguillarum (NCMB 6)	−	−	−	−
V. anguillarum (V-775)	−	−	−	−
Escherichia coli (Es-1)	−	−	−	−

* Autoagglutinative.

Table 2. Reaction of MAb-27 with fish pathogens by colony blotting test and FAT

Bacterial antigen used	Colony blotting test	FAT
Aeromonas hydrophila (IAM 1018)	−	−
A. hydrophila (*A. liquefaciens* EFDL)	−	−
A. hydrophila (TUF No. 1)	−	−
A. hydrophila (*A. punctata* NCMB 74)	−	−
A. salmonicida subsp. *salmonicida* (ATCC 14174)	+	+
A. salmonicida subsp. *salmonicida* (NCBM 1122)	+	+
A. salmonicida subsp. *salmonicida* (Shiga-1970)*	+	+
A. samlonicida subsp. *salmonicida* (Nagano-4175)	+	+
A. salmonicida subsp. *salmonicida* (France)	+	+
A. salmonicida subsp. *salmonicida* (Germany)	+	+
A. salmonicida subsp. *salmonicida* (Hokkaido-79388)	+	+
A. salmonicida subsp. *salmonicida* (Hokkaido-79389)	+	+
A. salmonicida subsp. *salmonicida* (Hokkaido-79385)	+	+
A. salmonicida subsp. *salmonicida* (Hokkaido-8825)*	+	+
A. salmonicida subsp. *salmonicida* (Hokkaido-8828)*	+	+
A. salmonicida subsp. *salmonicida* (Hokkaido-8828B)*	+	+
A. salmonicida subsp. *salmonicida* (Hokkaido-8834)*	+	+
A. salmonicida subsp. *salmonicida* (Hokkaido-8834B)*	+	+
A. salmonicida subsp. *salmonicida* (Hokkaido-8833)*	+	+
A. salmonicida subsp. *salmonicida* (Hokkaido-8835)*	+	+
A. salmonicida subsp. *achromogenes* (NCMB 1110)	+	+
A. salmonicida subsp. *masoucida* (NCMB 2020)	+	+
A. salmonicida subsp. *masoucida* (2-b-1)	+	+
Atypical *A. salmonicida* (Hokkaido-Carp)	+	+
Atypical *A. salmonicida* (Hokkaido-Carp)	+	+
Atypical *A. salmonicida* (Holland-Goldfish)	+	+
Aeromonas sp. (*pseudosalmonicida* No. 1)	−	−
Aeromonas sp. (*pseudosalmonicida* No. 2)	−	−
Vibrio anguillarum (NCMB-6)	−	−
V. anguillarum (V-775)	−	−
Escherichia coli (Es-1)	−	−

* Autoagglutinative.

Tabie 3. Maximum dilution of MAb-27 able to detect *A. salmonicida* subsp. *salmonicida* ATCC 14174 antigen by agglutination test, ELISA, FAT, and colony blotting test

Dilution antibody	Agglutination test*1	ELISA*2	FAT*1	Colony blotting test*1
1:1	++	+++	++	++
1:2	++	+++	++	++
1:4	++	+++	++	++
1:8	+	+++	+	++
1:16	−	+++	−	++
1:32	−	+++	−	++
1:64	−	+++	−	++
1:128	−	+++	−	+
1:256	−	++	−	−
1:512	−	−	−	−
1:1024	−	−	−	−

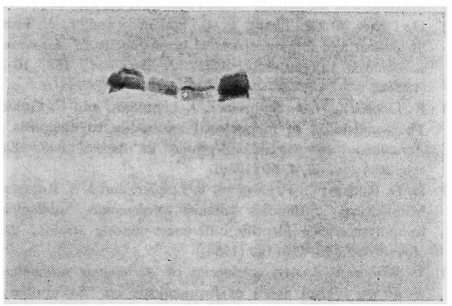
*1 +, Weakly positive; ++, strongly positive.

*2 ELISA absorbance: +, higher than 0.010; ++, higher than 0.050; and +++, higher than 0.070.

Table 4. Effects of the antigen concentration on the reaction of ELISA and colony blotting test using MAb-27 to detect the *A. salmonicida* subsp. *salmonicida* ATCC 14174 antigen

Number of bacteria (CFU/m*l*)	ELISA*	Colony blot*
10^8	++	++
10^7	+	+
10^6	−	+
10^5	−	−
10^4	−	−
10^3	−	−
10^2	−	−
10^1	−	−

* See Table 3.



A B C D E F G H

Fig. 1. Western blot analysis of cellular proteins of 7 bacteria employed, stained with immunoperoxidase using MAB-27 against *A. salmonicida*.
Lane A: Molecular weight markers (Sigma). Lane B: *A. salmonicida* subsp. *salmonicida* (ATCC 14174). Lane C: *A. salmonicida* subsp. *salmonicida* (NCMB 1122). Lane D: *A. salmonicida* subsp. *salmonicida* (Shiga). Lane E: *A. salmonicida* subsp. *masoucida* (NCMB 2020). Lane F: *A. hydrophila* (NCMB 86). Lane G: *A. hydrophila* (*A. punctata* NCMB 74). Lane H: *A. hydrophila* (*A. liquefaciens* EFDL).

and the colony blotting method. This MAbs showed a positive reaction by the agglutination test, ELISA, FAT, and the colony blotting test at maximum dilutions of 1:8, 1:256, 1:8, and 1:128, respectively (Table 3).

Sensitivity of MAb

Effects of the antigen consentration on the reaction of ELISA and colony blotting tests to detect the *A. salmonicida* ATCC 14174 angtigen using MAb-27 were observed. As shown in Table 4, the minimum concentration of the antigen showing a positive reaction was 10^7 and 10^6 CFU/m*l* for ELISA and the colony blotting test respectively.

Determination of the Antigen Recognized by MAb-27

Cellular proteins of 7 bacteria in SDS-PAGE gel were transferred to nitrocellulose membrane by western blotting and stained with MAb-27. The monoclonal antibody MAb-27 reacted with the proteins that originated from 3 strains of *A. salmonicida* subsp. *salmonicida* and 1 strain of *A. salmonicida* subsp. *masoucida*. No cross-reaction with other bacterial proteins was observed (Fig. 1). Molecular weights of these proteins were 51 to 62 kDa.

Discussion

In this study, we were able to establish 20 hybridomas to produce a monoclonal abtibody against *A. salmonicida* subsp. *salmonicida*. From these hybridomas, 4 were selected and tested for their specificity. Three of these hybridomas produced strain-specific antibodies and only one hybridoma (No. 27) produced a species-specific antibody. The characteristics of this MAb-27 are summarized as follows. The reaction was species-specific and reacted with *A. salmonicida* subsp. *salmonicida*, subsp. *achromogenes*, subsp. *masoucida*, and atypical *A. salmonicida*. The titer of this MAb was 1:8, 1:256, 1:8, and 1:128 for agglutination test, ELISA, FAT and colony blotting test, respectively. Molecular weights of cellular proteins, the recognized antigen of this MAb, were 51 to 62 kDa. The immunoglobulin subclass of MAb 27 was IgG2a and IgG3. These subclasses could not be separated by cloning and transplanting. These hybridomas might be fused by 2 spleen cells with one myeloma.

Other MAbs against components of the *A. salmonicida* cell surface have been reported.[10-12] Chart *et al.*[10] and Rockey *et al.*[12] reported that they had established a monoclonal antibody against *A. salmonicida* LPS and compared the structure and immunogenicity of different isolates of *A. salmonicida*. Rockey *et al.* showed that their MAbs were used to identify LPS heterogeneity within the species and the reactivity patterns were different among the different subspecies of *A. salmonicida*.[12]

Our MAb-27 reacted with *A. salmonicida* subsp. *salmonicida*, *achromogenes*, *masoucida*, and atypical *A. salmonicida* by slide aggluti-

nation test, ELISA, FAT, and colony blotting test. *A. salmonicida* subsp. *masoucida* was first isolated from diseased masu and pink salmon[13] and have not been isolated again until now. This monoclonal antibody has the ability to react with the antigen by several immunological methods routinely used in the laboratory. This monoclonal antibody might be useful for serological diagnosis of furunculosis including a subsp. *masoucida* infections and infections caused by atypical *A. salmonicida*. Furthermore, this might also be useful in detecting *A. salmonicida* antigen for the colony blotting method.

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