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Short communication

# Development of Anti-atypical *Aeromonas salmonicida* Monoclonal Antibodies for Diagnosis of "New Ulcer Disease" in Koi Carp

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**ABSTRACT**—To develop an accurate diagnosis of "new ulcer disease" in koi carp, we produced four monoclonal antibodies (mAbs) against a strain of atypical *Aeromonas salmonicida* isolated from koi carp. These mAbs did not cross-react with an isolate of atypical *A. salmonicida* from Japanese flounder and other pathogenic bacteria. Re-isolation from artificially infected koi carp was achieved by selecting the blue colonies on agar medium containing Coomassie brilliant blue, and some of the colonies were detected by immunofluorescent staining using the mAbs. These results suggested that the mAbs can distinguish atypical *A. salmonicida* from koi carp from resident aeromonads.

**Key words:** atypical *Aeromonas salmonicida*, monoclonal antibody, "new ulcer disease", koi carp

Ulcer disease causes progressive erosion of the skin and fins, resulting in high mortality and devastating disfigurement of the ornamental fishes. Because the disfigurement remarkably decreases its commercial value, this disease is one of the most serious concerns in koi carp aquaculture (Hunt, 2006). Therefore, it is important to establish a rapid and accurate diagnosis of ulcer disease in koi carp.

The atypical type of *Aeromonas salmonicida* was identified as a causative agent of "new ulcer disease" of koi carp (Matoyama *et al.*, 1999). Because the atypical *A. salmonicida* strains grow slowly on agar medium and are overtaken by the relatively fast-growing environmental bacteria, isolating them from fish showing symptoms of ulcer disease is often unsuccessful (Goodwin and Merry, 2009). Thus, to establish an accurate diagnosis of ulcer disease, it is necessary to isolate atypical *A. salmonicida* strains without contamination from other bacteria and to distinguish them from the resident aeromonad bacteria. We herein produced monoclonal antibodies (mAbs) against an isolate of atypical *A. salmonicida* from koi carp. Furthermore, the diagnosis of new ulcer disease in koi carp was established using a combination of detection by mAb and colony selection on agar medium containing Coomassie brilliant blue (CBB).

## Materials and Methods

Six isolates of atypical *A. salmonicida* from koi carp (T1031, T183, T01102, B12451, B10731) and common carp (B10F21) suffering from new ulcer disease was obtained from the Niigata Prefectural Inland-water Fisheries Experimental Station (Matoyama *et al.*, 1999; Gan *et al.*, 2015; Nakajima *et al.*, 2015; personal communication). *A. hydrophila* (CB-1 strain, Kusuda and Takahashi, 1970), an atypical *A. salmonicida* strain, isolated from Japanese flounder (TC63 strain, Iida *et al.*, 1997), *Flavobacterium columnare* (EK28 strain, Wakabayashi *et al.*, 1970) and *Edwardsiella tarda* (SH82195 strain, unpublished data) were purchased from the National Research Institute of Aquaculture. The bacterial culture method for each bacteria type was performed as described in each reference. In addition, formalin-inactivation of the bacteria was performed as previously described (Gan *et al.*, 2015).

BALB/c mice were intraperitoneally immunised with formalin-inactivated atypical *A. salmonicida* (T1031) after it had been emulsified with an equal volume of Freund's complete adjuvant. Booster injections were administered three times at intervals of 2 weeks using the emulsion with Freund's incomplete adjuvant. Monoclonal antibody (mAb) was essentially produced as previously described (Hirose *et al.*, 2005). The hybridoma in the positive well was cloned twice by the limiting dilution technique, and the representative cell clones were used for the preparation of the mAb. Hybridoma screening for specific reactivity to immunogen was determined by ELISA or fluorescent antibody technique

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(FAT) (Gan *et al.*, 2015). Sandwich ELISA was performed with biotinylated second mAbs and alkaline phosphatase-conjugated streptavidin as previously described (Hirose *et al.*, 2005). Animal experiments were performed in agreement with the guidelines of the Animal Experiment Committee of Kyoto Women's University.

To confirm whether or not the mAbs are derived from distinct clones, cDNA fragments encoding variable regions of heavy and light chains ($V_H$ and $V_L$, respectively) from the hybridomas were sequenced as previously described (Sato *et al.*, 2005). In brief, total RNA from hybridoma cells was reverse-transcribed using the SMART$^{TM}$ RACE cDNA Amplification Kit (Clontech). The cDNA fragments of the $V_H$ and $V_L$ regions were generated by PCR using isotype-specific primers that have been previously reported (Sato *et al.*, 2005). The PCR products were cloned into the pGEM-T Easy vector (Promega) and confirmed by DNA sequencing analysis.

Bath infection procedure of *A. salmonicida* (T1031) was followed as previously described (Gan *et al.*, 2015). In brief, five koi carp, weighing 2.5–4.5 g, were exposed to $3 \times 10^6$ CFU/mL of *A. salmonicida* for 1 h at 20°C in a 1-L tank. Subsequently, they were transferred to 45-L tanks which did not contain the bacteria, and disease symptoms were observed every day. The fish that displayed ulcers on the skin and/or fins were sampled, and the bacteria on their affected areas were collected and streaked on heart infusion agar (HI; Nissui) containing CBB (0.1 mg/mL) by platinum loop. After the plate was incubated for 4–5 days, blue and non-blue colonies were selected and transferred to HI broth. The suspension of the propagated bacteria was centrifuged at 500 $\times g$ for 10 min, and the bacteria were re-suspended in PBS. The bacteria were incubated with anti-*A. salmonicida* mAb (8H) for 30 min, washed twice with PBS and then incubated with Alexa Fluor 488-labelled goat anti-mouse immunoglobulin G (IgG; diluted 1/200, Life Technologies) on ice for 30 min. Labelled bacteria were washed twice with PBS and observed by a fluorescent microscope. The bacteria stained with only secondary antibody were used as negative controls. In addition, fluorescence intensity of the bacteria was analysed with an Attune NxT Flow Cytometer (Thermo Fisher Scientific).

To genetically identify whether or not the isolated bacteria are atypical *A. salmonicida*, PCR was performed using two sets of *A. salmonicida*-specific primers as previously described (Nilsson *et al.*, 2006). ISAsa4-primers and AP-primers were used to detect only the atypical strain and both atypical and typical strains, respectively.

## Results and Discussion

Four hybridoma clones, designated 8H, 4A, 5H and 2B (IgG2b) were established from mice immunised with the isolate of *A. salmonicida* from koi carp. All mAbs from four distinct hybridomas bound the atypical *A. salmonicida* T1031 strain (immunogen) but not *A. hydrophila, F. columnare, E. tarda* and the isolate of atypical *A. salmonicida* from flounder, showing no cross-reactivity against the other fish pathogenic bacteria (Fig. 1A). Isolates from koi carp (T183, T01102, B12451, B10731) and common carp (B10F21) could be detected by FAT using the mAbs (data not shown). In sandwich ELISA, several combinations of the mAbs, such as 4A and 8H, 4A and 9A and 2B and 2B (coated and labelled

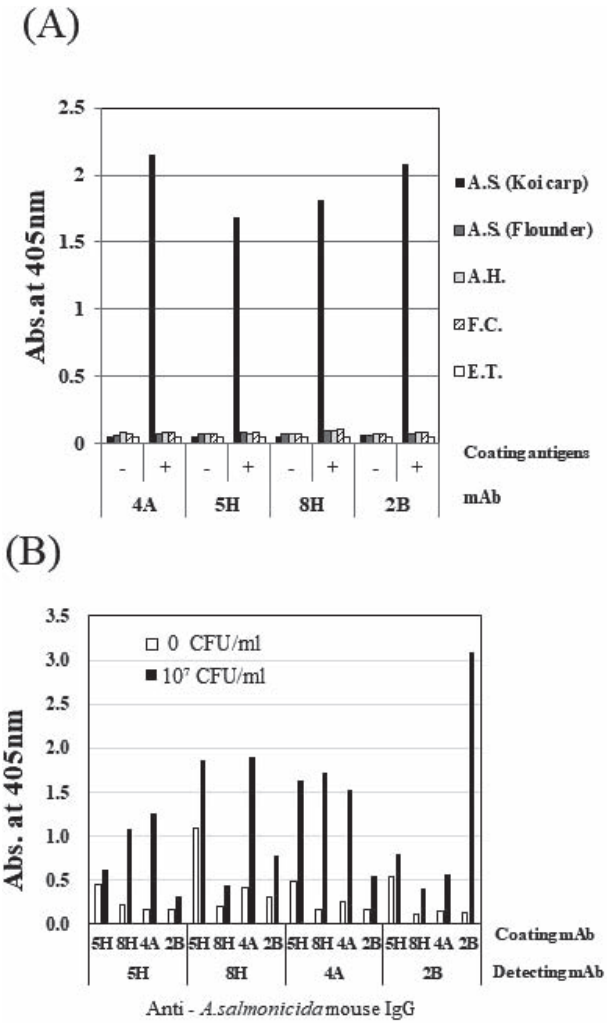


**Fig. 1.** (A) Cross-reactivity of anti-*Aeromonas salmonicida* mAb (4A, 5H, 8H, 2B) to isolates of atypical *A. salmonicida* (*A.S.*) from flounder, *A. hydrophila* (*A.H.*), *Flavobacterium columnare* (*F.C.*) and *Edwardsiella tarda* (*E.T.*). The formalin-killed bacteria were coated on 96-well plates (+). Uncoated wells were used as negative controls (–). (B) Sandwich ELISA using the mAb to detect atypical *A. salmonicida*. Clone names in the upper row on the X-axis specify mAbs that were coated onto 96-well plate, and the lower clones show biotinylated mAbs used as detecting antibodies. An isolate from atypical *A. salmonicida* in koi carp ($10^7$ CFU) was used as an antigen.

mAb, respectively), could detect *A. salmonicida* with high sensitivity (Fig. 1B).

$V_H$ and/or $V_L$ sequences from four mAbs were not identical (4A-$V_H$, LC225752; 8H-$V_H$, LC225753; 5H-$V_H$, LC225754; 4A-$V_L$, LC225755; 8H-$V_L$, LC225756; 2B-$V_L$, LC225757), indicating that they are distinct antibodies. Although the antigenic epitopes of the mAb could not be identified in the present study, they presumably recognise different epitopes on the bacteria. Atypical *A. salmonicida* has been isolated from many species in both marine and freshwater fish and has been classified into four groups based on nucleic acid sequence of 16S rDNA (Yamada *et al.*, 2000). Although several studies have reported that anti-*A. salmonicida* sera are available (Kaku *et al.*, 1999), polyclonal antibodies have failed to discriminate subgroups. Because these mAbs did not cross-react to an isolate of atypical *A. salmonicida* from flounder, they may more precisely detect the isolates from koi carp. However, we could not investigate reactivity of the mAbs against isolates from fish species except carp and flounder in this study. A previous study showed that biological characteristics of the isolates from new ulcer disease of carp are different from those of atypical *A. salmonicida* from ulcer disease of goldfish (Matoyama *et al.*, 1999). Thus, to confirm availability of the mAbs in diagnosis of new ulcer disease, we need to examine the reactivity of the mAbs against isolates from goldfish and eel.

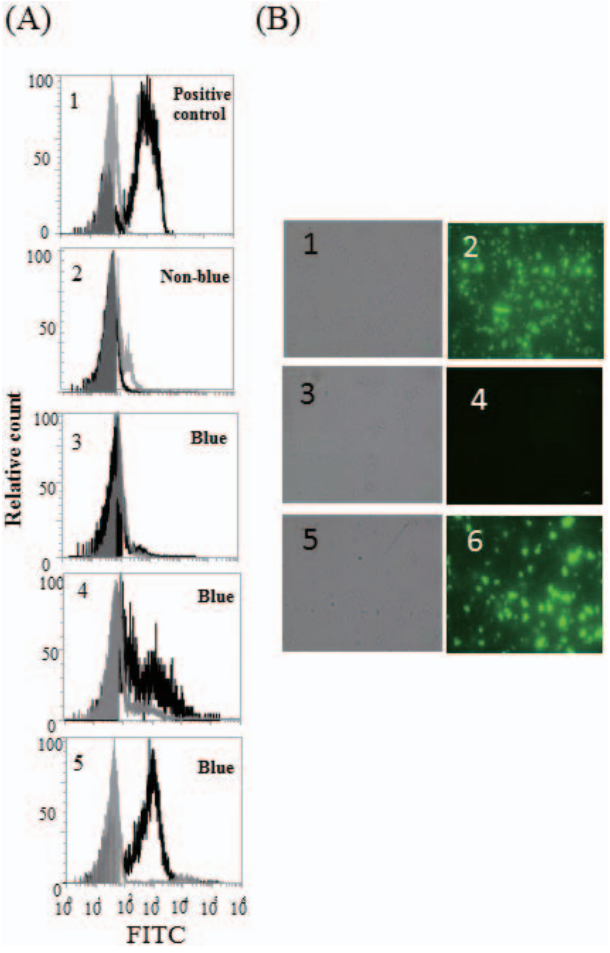


**Fig. 2.** (A) Flow cytometry analysis of atypical *Aeromonas salmonicida* T1031 (1), non-blue colony bacteria (2) and blue colony bacteria (3–5). Black and grey signals indicate mAb (8H)-stained and unstained bacteria, respectively. (B) Fluorescent microscope observation of *A. salmonicida* T1031 (1 and 2) and the mAb-negative (3 and 4) and mAb-positive bacteria (5 and 6). The pictures on the left (1, 3 and 5) and right lanes (2, 4 and 6) show bright and fluorescent (FITC) fields, respectively.

Fish that have skin and fin ulcers were observed 4 days after infection. Bacteria were isolated from the diseased fish, and selected bacteria formed blue colonies on the CBB HI agar. Positive fluorescent signals of bacteria forming blue colonies were detected in the mAb-treated bacteria by fluorescent microscope observation and FCM analysis, whereas one of the three colonies was negative. No positive signal was detected in the negative control and non-blue colony bacteria (Fig. 2A and B). Expected size (ISAsa4; 749 bp, AP; 421 bp) of amplified DNA fragments were observed by PCR in the mAb-positive bacteria (Fig. 3). The PCR product that was observed in the mAb-negative bacteria displayed a slightly higher band than expected (lane 2 in ISA). Previous reports have shown that CBB is a protein-binding dye that binds the A-layer of *A. salmonicida*, indicating that the selection by CBB agar was useful for screening of *A. salmonicida* (Cipriano and Bertolini, 1988). However, this blue colony selection is not always reliable in primary isolation (Teska and Cipriano, 1993), as we picked up mAb-negative bacteria that had formed blue colonies. Thus, the identification by the mAb is essential for secondary screening.

Although we detected *A. salmonicida* using immunofluorescent methods in the present study, the detection system using mAbs can be applicable to that using

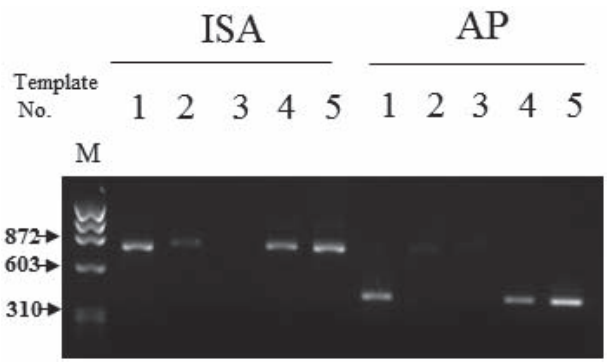


**Fig. 3.** PCR analysis of isolated bacteria using ISAsa4-primers (ISA) and AP-primers (AP). Expected amplified products are 749 bp (ISA) and 421 bp (AP). M: marker ($\varphi$X174 DNA-Hae III Digest; Takara); template 1: positive control (*Aeromonas salmonicida* T1031); template 2: mAb-negative bacteria; template 3: mAb-negative bacteria; template 4: mAb-positive bacteria; template 5: mAb-positive bacteria.

enzyme stain. The rapid diagnostic test (RDT) kits using enzyme stain, such as the RDT-kit for influenza virus, could be made available for ulcer diagnosis in koi farms and institutes that do not have a fluorescent microscope. Thus, ulcer diagnosis using the mAb from the present study can be practically utilised and may contribute to preventing ulcer disease in koi carp aquaculture.

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## References

Cipriano, R. C. and J. Bertolini (1988): Selection for virulence in the fish pathogen, *Aeromonas salmonicida*, using Coomassie Brilliant Blue agar. *J. Wild. Dis.*, **24**, 672–678.

Gan, H., H. He, A. Sato, H. Hatta, M. Nakao and T. Somamoto (2015): Ulcer disease prophylaxis in koi carp by bath immersion with chicken egg yolk containing anti-*Aeromonas salmonicida* IgY. *Res. Vet. Sci.*, **99**, 82–86.

Goodwin, A. E. and G. E. Merry (2009): Are all koi ulcer cases associated with infection by atypical *Aeromonas salmonicida*? Polymerase chain reaction assays of koi carp skin swabs submitted by hobbyists. *J. Aquat. Anim. Health*, **21**, 98–103.

Hirose, J., Y. Murakami-Yamaguchi, M. Ikeda, N. Kitabatake and H. Narita (2005): Oligoclonal enzyme-linked immunosorbent assay capable of determining the major food allergen, ovomucoid, irrespective of the degree of heat denaturation. *Cytotechnology*, **47**, 145–149.

Hunt, C. J. (2006): Ulcerative skin disease in a group of koi carp (*Cyprinus carpio*). *Vet. Clin. North Am. Exot. Anim. Pract.*, **9**, 723–728.

Iida, T., C. Sakata, H. Kawatsu and Y. Fukuda (1997): Atypical *Aeromons salmonicida* infection in cultured marine fish. *Fish Pathol.*, **32**. 65–66. (in Japanese with English summary)

Kaku, Y., Y. Yamada and H. Wakabayashi (1999): Characterization of atypical *Aeromonas salmonicida* isolated from an epizootic ulcerative disease in carp (*Cyprinus carpio*). *Fish Pathol.*, **34**, 155–162. (in Japanese with English summary)

Kusuda, R. and Y. Takahashi (1970): Studies on the scale protrusion disease of carp fishes-I. Characteristics of *Aeromonas liquefaciens* isolated from diseased fishes. *Fish Pathol.*, **4**, 87–97. (in Japanese with English summary)

Matoyama, H., M. Hoshino and H. Hosoya, (1999): Pathogenicity of atypical *Aeromonas salmonicida* isolated from colored carp (*Cyprinus carpio*) suffering from a new ulcerative disease. *Fish Pathol.*, **34**, 189–193. (in Japanese with English summary)

Nakajima, K., R. Yoshizawa and N. Hyodo (2015): The drug sensitive of atypical *Aeromonas salmonicida* isolated from the nishikigoi, *Cyprinus carpio*, suffering from ulcer disease. *Rep. Niigata Pref. Inland Wat. Fish Exp.*, **39**, 1–3. (in Japanese)

Nilsson W. B., N. Gudkovs, M. and S. Strom (2006): Atypical strains of *Aeromonas salmonicida* contain multiple copies of insertion element ISAsa4 useful as a genetic marker and a target for PCR assay. *Dis. Aquat. Organ.*, **70**, 209–217.

Sato, M., R. Iwaya, K. Ogihara, R. Sawahata, H. Kitani, J. Chiba, Y. Kurosawa and K. Sekikawa (2005): Intrabodies against the EVH1 domain of Wiskott-Aldrich syndrome protein inhibit T cell receptor signalling in transgenic mice T cells. *FEBS J.*, **272**, 6131–6144.

Teska, J. D. and R. C. Cipriano (1993): Nonselective nature of Coomassie Brilliant Blue agar for the presumptive identification of *Aeromonas salmonicida* in clinical specimens. *Dis. Aquat. Organ.*, **16**, 239–242.

Wakabayashi, H., K., Kira and S. Egusa (1970): Studies on columnaris disease of pond-cultured eels-I. Characteristics and pathogenicity of *Chondrococcus columnaris* isolated from pond-cultured eels. *Nippon Suisan Gakkaishi* **36**, 147–155. (in Japanese with English summary)

Yamada Y., Y. Kaku and H. Wakabayashi (2000): Phylogenetic Intrarelationships of Atypical *Aeromonas salmonicida* Isolated in Japan as Determined by 16S rDNA Sequencing. *Fish Pathol.*, **35**, 35–40.