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Rapid Method for Detecting Pathogenic *Aeromonas caviae* in Fish using Monoclonal Antibodies on Test Strips

Yidan Huang[a], Kaiyu Wang[a]*, Jian Zheng[b], Jinlu Huang[a], Yi Geng[a]

[a] Research Center of Fish Disease, Sichuan Agricultural University, Ya'an, Sichuan 625014, China

[b] Key Laboratory of Molecular Biology on Infectious Disease, Chongqing Medical University, Chongqing 400016, China

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Abstract

Aeromonas caviae is a pathogenic bacteria that causes disease in a variety of animals, especially fish. In this study, we prepared strips of monoclonal antibody (mAb), conjugated to colloidal gold, as a rapid diagnostic method for detecting antigens to *A. caviae*. BALB/c mice, an albino, laboratory-bred strain of the House Mouse from which a number of common substrains are derived, were divided into two groups. One group was immunized with 25 µg formalin-killed *Aeromonas caviae*; the second group with 50 µg. Hybridoma cell lines that consistently secrete mAbs against *A. caviae* were obtained by cell fusion. Two strains of hybridomas (3F3 and 2C9C3) were screened. Subtypes of the two mAbs were IgG_1 and IgM, confirmed by indirect enzyme linked immunosorbent assay (ELISA). Titers of 3F3 and 2C9C3 mAb in ascite fluid were $1:10^6$ and $1:10^5$, respectively. The strains targeted different antigen epitopes: 3F3 targeted the lipopolysaccharide (LPS) of *A. caviae*, while 2C9C3 targeted non-LPS sites. We tested our gold colloidal strips on the hepatopancreas, kidney, and spleen of bacteria-challenged common carp (*Cyprinus carpio*). Specificity, sensitivity, repeatability, and clinical tests showed the strips can effectively and rapidly identify pathogenic *A. caviae* in aquacultured fish.

* Corresponding author. Tel.: 860835-2885753, fax: 860835-2885302, e-mail: kywang@sicau.edu.cn

Introduction

Motile *Aeromonas* species are widely distributed in nature and normal microbial flora in aquatic and terrestrial organisms (Trust and Sparrow, 1974; Kaper et al., 1981). Species have been isolated from local and generalized human infections (Chaudhury et al., 1996; Ko et al., 2000). Many members of the group are primary pathogens to a range of poikilothermic animals, in particular, fish (Aoki et al., 1971; Son et al., 1997).

The genus *Aeromonas* is a major causative agent of infections in fish (Austin and Adams, 1996). *Aeromonas caviae*, one of the most prominent pathogenic bacteria in China, is harmful to the freshwater aquaculture industry (Nielsen et al., 2001). Our research center first reported a southern catfish epizootic ulcerative syndrome associated with *A. caviae* in 2008 (Ji et al., 2008). Fish infected with *A. caviae* develop disease with symptoms such as septicemia, enteritis, body surface ulcers, and lepidorthosis. Diseases caused by *A. caviae* are characterized by rapid invasion, wide prevalence, and high mortality, causing fatalities in aquatic animals and infections in humans. *Aeromonas caviae* can cause cystitis (Al-Benwan et al., 2007), inflammatory bowel disease (Van der Gaag et al., 2005), and bacteremia (Teira et al., 1991).

Commonly used methods of diagnosing *A. caviae* include clinical observation, dissection, and common bacteriology tests that are time-consuming and not always accurate. The establishment of a rapid method with higher specificity would better identify and control infections caused by *A. caviae*. Since the development of procedures for monoclonal antibody (mAb) production (Kohler and Milstein, 1975), mAbs have been widely used in clinical diagnosis. One promising assay format involves test strips of mAbs conjugated to colloidal gold to detect antigens. In this study we prepared anti-*A. caviae* mAbs, identified their biological characteristics, and developed colloidal gold test strips as a rapid and accurate diagnostic method for *A. caviae* in aquaculture.

Materials and Methods

Preparation of bacteria. A southern catfish (*Silurus meridionalis* Chen) affected by epizootic ulcerative syndrome was obtained from an aquaculture facility in Sichuan Province, China, in 2005. Based on morphological, physiological, biochemical characteristics, and sequence analysis of 16S rDNA (Ji et al., 2008), the catfish was infected by *A. caviae*. Another strain of the bacteria was obtained from the American Type Culture Collection (ATCC), Research Center of Fish Disease and Key Laboratory of Molecular Biology on Infectious Disease in China, for use in cross-reaction testing. The bacteria were cultured at 28°C in tryptic soy broth (TSB) and harvested by centrifugation at 3500 × *g* for 20 min at 4°C. Bacteria pellets were washed twice, then suspended in physiological saline. Bacterial proteins were quantified according to Bradford (1976), with bovine serum albumin as the standard protein.

Hybridoma generation. Formalin-killed *A. caviae* were emulsified in an equal volume of Freund's complete adjuvant (FCA; Sigma) at doses of 25 μg

and 50 μg bacterial proteins, and female BALB/c mice, an albino, laboratory-bred strain of the House Mouse from which a number of common substrains are derived, were injected subcutaneously to generate hybridomas. Two weeks after immunization, the mice were re-immunized subcutaneously with the same amount of protein and Freund's incomplete adjuvant. The step was repeated twice, followed by a caudal intravenous booster injection of 50 μg protein in phosphate buffered solution (PBS) before cell fusion.

Splenocytes of the immunized mice were harvested three days after the final booster injection. These cells were fused with myeloma cell line SP2/0 at a ratio of 5:1 with 50% (w/v) polyethyleneglycol 4000 (Sigma; Situ and Wu, 1996). Fusion products were selected in hypoxanthine, aminopterin, and thymidine (HAT) tissue culture medium according to Littlefield (1964). Mouse peritoneal cells were used as feeder cells. Hybridomas were cultured in RPMI1640 medium (Hyclone, Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS; Hyclone). The hybridoma supernatants were screened by indirect enzyme linked immunosorbent assay (ELISA). The OD_{450} value was recorded and positive cells were screened (positive/negative>2; Zhang et al., 2002). Positive hybridomas were cloned by the soft agar cloning method (Situ and Wu, 1996) and selected according to the characterization of the secreted mAbs. Ascites were collected and the anti-*A. caviae* mAbs in the ascites were purified by Protein G Sepharose affinity chromatography (Sigma; Wang and Fan, 2002).

Specificity, subtype, titer, and relative affinity analysis of mAbs. Specific mAbs were screened by indirect ELISA, using six bacterial strains. A 96-well microtiter plate was coated with 10 μg/ml of formalin-killed antigen. Positive serum of immunized mice was used as the positive control and Sp2/0 supernatant as the negative control. 100 μl of the hybridoma supernatant was added to the wells and kept at 37°C for 1 h. The wells were treated with 100 μl peroxidase-conjugated goat anti-mouse IgG diluted by PBS (1:10000), then stained by tetramethyl benzidine substrate (TMB substrate), and the optical density of each well was recorded at 450 nm with a Synerg HT Multi Detection Microplate Reader (BIOTEK).

Following manufacturer's instructions, the class and subclass of the anti-*A. caviae* mAbs were determined using indirect ELISA, with specific antibodies in six isotypes: goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA (supplied by Sigma Immunochemicals, Iso-2). The ascites in different dilutions were measured by indirect ELISA to determine the titer of ascites. Then according to the optical density of each well as recorded at 450 nm, a standard curve was drawn according to the OD_{450} value. The value on the flat upside of the curve was considered 100% and 50% of the OD_{450} value was considered the relative affinity of the mAbs (Friguet et al., 1983; Su et al., 2002).

Identification of epitopes. Additive ELISA was conducted according to Friguet et al. (1983) and OD_{450} values were recorded. The added index (AI) was determined by the formula $[(2A1+2/(A1 + A2)) - 1] \times 100\%$, where A1 and A2 are the OD_{450} values of the two mAbs, respectively, and A1+2 is the

OD_{450} value of the two mAb mixture. If AI>40%, then the two mAbs recognize the different antigenic sites, otherwise they recognized the same site. (Gan et al., 1999). Indirect ELISA and the extraction of lipopolysaccharide (LPS) of *A. caviae* was conducted according to Carlson et al. (1987) and Carrrion et al. (1990). A 96-well microtiter plate was coated with 5 µg/ml LPS of *A. caviae*, 100 µl of purified ascites of 3F3 or 2C9C3 were added to the wells, the wells were treated with 100 µl peroxidase-conjugated goat anti-mouse IgG diluted by PBS (1:10000), the contents were stained with TMB substrate, and the optical density of each well was recorded at 450 nm, as above.

Mixture of anti-A. caviae mAb and colloidal gold. 0.2 ml of pure mAb (concentration 2 mg/ml) were added to 100 ml colloidal gold liquid, the mixture was kept at room temperature for 2 min, 0.2% polyethylene glycol (PEG) was added, and the solution was centrifuged by 4000 × *g* for 30 min. The red deposition was collected in a 12-ml centrifuge tube, centrifuged at 8000 × *g* for 30 min, suspended with the colloidal gold torpent, and diluted into the working concentration (commonly OD_{532} = 30-40). The solution was kept at 4°C.

Reaction membrane M1. The mAb was diluted to 1.6 mg/ml with 0.1 M PBS. Goat anti-mouse IgG was diluted to 1.0 mg/ml with 0.1 M PBS. The former was used to coat the test line, while the latter to coat the control line, on nitrocellulose membrane (NC). The M1 was kept at 37°C for 24 h. After being blocked for 30 min in BB buffer (Artron BioResearch Inc, Canada), the M1 was washed with WB buffer (Artron BioResearch Inc, Canada) and kept at 37°C.

Reaction membranes M2a, M2b, M3, and M4, and assembly of strips. Fibrous membranes (Artron BioResearch Inc, Canada) were soaked in solutions 2a, 2b, or 3 (Artron BioResearch Inc, Canada), dried, and kept in plastic bags at room temperature. The colloidal gold/anti-*A. caviae* mAb mixture was sprayed on fibrous membrane M4 twice (0.5-1.5 × 25 cm/strip). After drying at 37°C for 12 h, the M4 was kept at room temperature. The membranes were assembled and cut into 4-mm pieces with an auto-stripcutter (Fig. 1).

Detection and sensitivity to A. caviae using strips. The pad of the strip was dipped into samples of *A. caviae* and formalin-killed *A. caviae* until half the NC membrane was soaked in the liquid. The color development of the control and test lines was observed after 3-10 min at room temperature. The *A. caviae* sample was then gradually diluted from 6.86 × 10^9 to 8.55 × 10^3 cfu/ml, and the strips were again tested to evaluate the minimum detection dose by counting the bacteria. Specificity was tested on samples of *Aeromonas caviae*, *A. caviae* (ATCC15468), *A. punctata*, *A.*

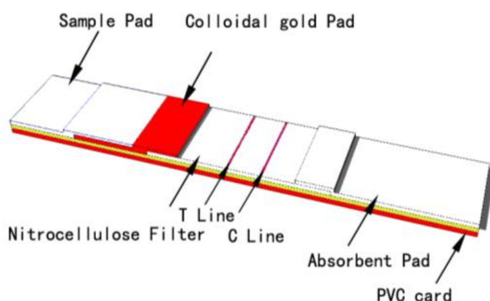

Fig. 1. Model of the strip.

hydrophila, *A. veronii*, *Streptococcus iniae*, *Yersinia ruckeri*, *Edwardsiella ictaluri*, *Staphylococcus aureus*, and *Stenotrophomonas maltophilia.* Three samples of *A. caviae* and three samples of the negative control were tested by the strips with ten replicates per sample to test for repeatability.

Test of pathological specimens. Twenty healthy common carp (*Cyprinus carpio*) were injected intraperitoneally with 0.1 ml *A. caviae* (3.5×10^8 cfu/ml) per fish; another 20 were injected with the same dosage of *A. hydrophila*. The hepatopancreas, kidney, and spleen of the challenged fish were gathered. The tissues were homogenized and centrifuged, and the supernatants kept for detection. Ascitic fluid from the challenged fish was used directly for detection. PBS was used as the negative control.

Results

Specificity of mAb. After cell fusion, three strains of hybridoma cell lines were collected that could secrete anti-*A. caviae* mAb: 2D5, 3F3, and 2C9C3. Strains 3F3 and 2C9C3 had high specificity, and results were confirmed by ELISA. Strain 2D5 cross-acted with *Aeromonas* spp. (Table 1).

Table 1. Cross-reactions of mAbs against bacterial genera (OD_{450} value, $\overline{X} \pm S$, $n = 3$).

mAb	*Bacteria*					
	Aeromonas caviae	*Aeromonas hydrophila*	*Aeromonas veronii*	*Streptococcus iniae*	*Yersinia ruckeri*	*Escherichia coli*
2D5	0.918±0.023	0.875±0.017	0.893±0.006	0.283±0.003	0.267±0.01	0.204±0.026
3F3	1.099±0.017	0.128±0.021	0.236±0.006	0.132±0.015	0.129±0.021	0.134±0.014
2C9C3	1.032±0.005	0.146±0.013	0.287±0.022	0.239±0.02	0.212±0.015	0.127±0.023
Negative	0.119±0.10					

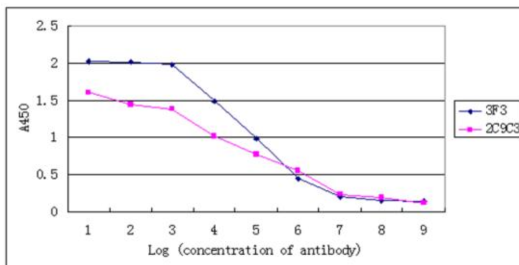

Fig. 2. Relative affinity analysis of mAb 3F3 and 2C9C3.

Subtypes, titers and relative affinity analysis of mAb. The subtypes of 3F3 and 2C9C3 were IgG_1 and IgM, respectively. After testing the titers, 3F3 was about 10^{-6} and 2C9C3 about 10^{-5}. The relative affinities of 3F3 and 2C9C3 were higher than 10^5, and 3F3>2C9C3 (Fig. 2).

Identification of epitopes. Additive ELISA showed that mAbs 3F3 and 2C9C3 recognize different antigenic sites (AI>40%; Table 2). Indirect ELISA showed that 3F3 is positive to the LPS of *A. caviae*, but 2C9C3 is negative (Table 3), meaning that 3F3 targets the LPS of *A. caviae*, while 2C9C3 targets non-LPS sites.

Detection of bacteria by strips. Whether killed by formalin or not, samples of *A. caviae* produced clear purple strips. The strip near the sample pad was the test line and the one near the label pad served as the control. The minimum detection dose of the strips was 1.71×10^4 cfu/ml (Fig. 3).

Table 2. Identification of epitopes by additive ELISA (OD_{450} value).

mAb	*3F3*	*2C9C3*
3F3	0.92	1.070 (AI=47.99%)
2C9C3	-	0.526

Table 3. Identification of epitopes by indirect ELISA (OD_{450} value).

	mAb		
	3F3	*2C9C3*	*Negative*
LPS of *A. caviae*	0.764	0.137	0.103

Aeromonas caviae was positive, while *A. caviae* (ATCC15468), *A. punctata*, *A. hydrophila*, *A. veronii*, *S. iniae*, *Y. ruckeri*, *S. maltophilia*, *S. aureus*, and *E. ictaluri* were negative (Fig. 4). The repeatability test showed that both the positive and the negative coincidence were 100%.

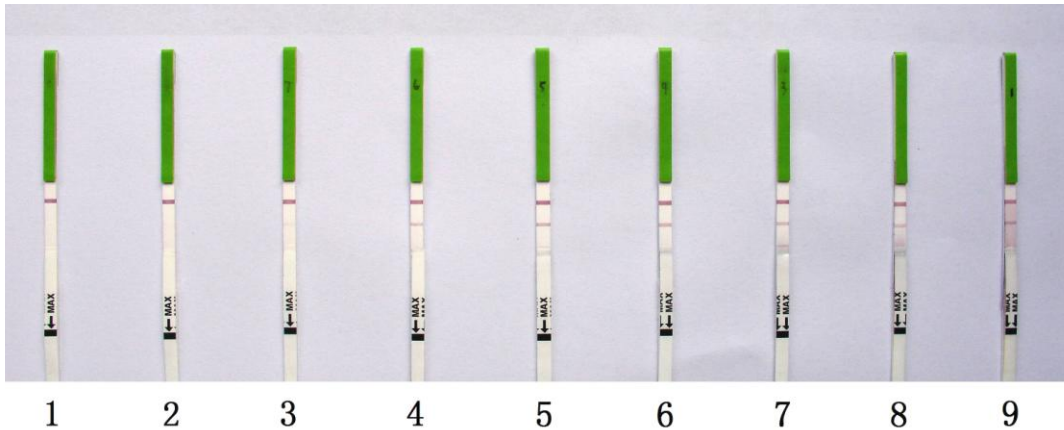

Fig. 3. Sensitivity test: 1 = 8.55×10^3 cfu/ml, 2 = 1.71×10^4 cfu/ml, 3 = 3.43×10^4 cfu/ml, 4 = 6.86×10^4 cfu/ml, 5 = 6.86×10^5 cfu/ml, 6 = 6.86×10^6 cfu/ml, 7 = 6.86×10^7 cfu/ml, 8 = 6.86×10^8 cfu/ml, and 9 = 6.86×10^9 cfu/ml.

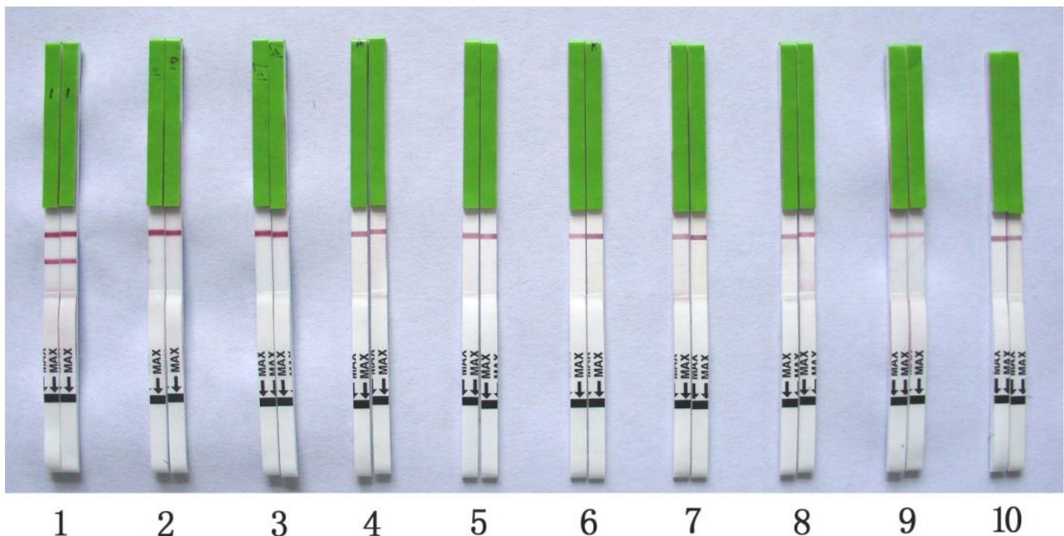

Fig. 4. Specificity test: 1 = *Aeromonas caviae*, 2 = *A. veronii*, 3 = *A. caviae* (ATCC15468), 4 = *A. punctata*, 5 = *Yersinia ruckeri*, 6 = *A. hydrophila*, 7 = *Stenotrophomonas maltophilia*, 8 = *Streptococcus iniae*, 9 = *Staphylococcus aureus*, and 10 = *Edwardsiella ictaluri*.

Results of pathological specimen test. Because there was no ascitic fluid in the *A. hydrophila* group, ascitic fluid was collected from the *A. caviae* group only. The ascitic fluid and supernatants of tissues from the *A. caviae* group were test-positive, while supernatants of tissues from the *A. hydrophila* group were test-negative (Fig. 5).

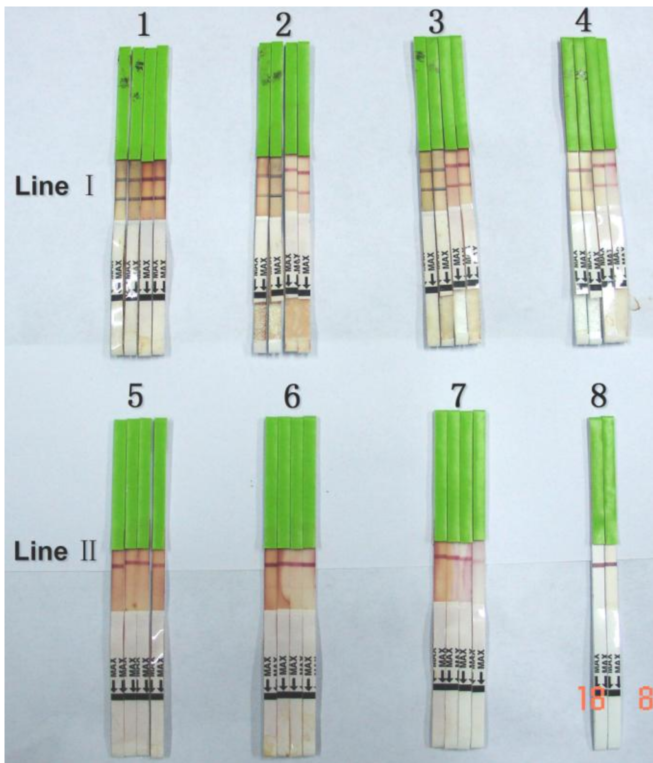

Fig. 5. Pathological specimen test in *Aeromonas caviae* group (Line I): 1 = ascitic fluid, 2 = hepatopancreas, 3 = spleen, 4 = kidney, and in *A. hydrophila* group (Line II): 5 = hepatopancreas, 6 = spleen, 7 = kidney, and 8 = PBS.

Discussion

Since the development of mAbs technology, mAbs are frequently used to diagnose disease, detect medicine residues, and for other purposes. The development of mAb for *A. caviae* has a promising future, especially since they can be prepared by molecular immunology with a high affinity for rapid diagnosis and epidemic surveillance. *Aeromonas caviae* is an opportunist pathogen in fish and can cause many aquatic animal diseases (Nielsen et al., 2001). In this study, two specific mAbs, 3F3 and 2C9C3, were prepared by the hybrid technique to develop a rapid detection method for *A. caviae* in aquaculture. The subtypes, titer, and specificity of the mAbs were analyzed and results show that these mAbs have high specificity, good stability, high titer, and relative affinity, and are thus adequate diagnostic agents. The two mAb strains targeted different antigen epitopes: 3F3 targeted the LPS of *A. caviae*, while 2C9C3 targeted the non-LPS sites. Understanding of the structure and function of antigen epitopes can help us learn more about immune reactions and antigen/antibody interactions. Such information can be used to develop safe effective vaccines and diagnostic agents based on antigen epitopes (Wang and Yu, 2004). The preparation of anti-*A. caviae* mAbs that target different antigen epitopes lays the foundation for the development of colloidal gold test strips based on the mAbs.

Colloidal gold technology is one of four immunomarker technologies that developed rapidly and are applied in all research fields of biomedicine, especially in medical laboratories (Bendayan, 2000). Colloidal gold combines with various biomacromolecules because of its high electron-density. It has became a common nonradioactive tracer for immunomarkers, after fluorescein, radioactive isotopes, and enzymes. Many colloidal gold strips have been developed to diagnose animal diseases.

Our anti-*A. caviae* colloidal gold strips are an inexpensive, rapid, and specific detection method, meaning that they have bright prospects for application in aquaculture. The strips are based on the double antibody sandwich method (mAb-antigen-mAb). *Aeromonas caviae* (ATCC15468) is a non-pathogenic strain, and was used to contrast with our pathogenic strain. The strip reacted with the pathogenic *A. caviae* and not with the non-pathogenic *A. caviae*. Further research is required to determine the difference between pathogenic and non-pathogenic *A. caviae*.

The minimum detection dose of the strips was 1.71×10^4 cfu/ml, which means it has high sensitivity. At present, the detection of *A. caviae* depends on morphologic observation, physiological and biochemical tests, and PCR (Ji et al., 2008). Utilization of colloidal gold strips is simpler and more convenient than these methods. Using the strips, results were observed within five minutes. The color of the test line is proportional to the content of *A. caviae* antigen: the color of the T Line deepens with an increase of *A. caviae* content. The specimen test showed that the strips can detect *A. caviae* in organs of diseased fish, proving that the strips can be used easily and exactly in clinical tests. As a result of our work, culturists can take immediate measures to curb the proliferation of *A. caviae*.

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References

Al-Benwan K., Abbott S., Janda J.M., Huys G. and M.J. Albert, 2007. Cystitis caused by *Aeromonas caviae*. *J. Clin. Microbiol.*, 45(7):2348-2350.

Aoki T., Egusa S., Ogata Y. and T. Watanabe, 1971. Detection of resistance factors in fish pathogen *Aeromonas liquefaciens*. *J. Gen. Microbiol.*, 69:343-349.

Austin B. and C. Adams, 1996. Fish pathogens. pp. 197-229. In: B. Austin, M. Altwegg, P.J. Gosling, S. Joseph (eds.). *The Genus Aeromonas*. John Wiley & Sons, New York.

Bendayan M., 2000. A review of the potential and versatility of colloidal gold cytochemical labeling for molecular morphology (Review). *Biotechnic & Histochem.*, 75(5):203-242.
Bradford M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72:248-254.
Carlson R.W., Kalembasa S., Turowski D., Pachori P. and K.D. Noel, 1987. Characterization of the lipopolysaccharide from a *Rhizobium phaseoli* mutant that is defective in infection thread development. *J. Bacteriol.*, 169(11):4923-4928.
Carrion M., Bhat U.R., Reuhs B. and R.W. Carlson, 1990. Isolation and characterization of the lipopolysaccharides from *Bradyrhizobium japonicum*. *J. Bacteriol.*, 172(4):1725-1731.
Chaudhury A., Nath G., Shukla B.N. and S.C. Sanyal, 1996. Biochemical characterisation, enteropathogenicity and antimicrobial resistance plasmids of clinical and environmental *Aeromonas* isolates. *J. Med. Microbiol.*, 44:434-437.
Friguet B., Djavadi-Ohaniance L., Pages J., Bussard A. and M. Goldberg, 1983. A convenient enzyme-linked immunosorbent assay for testing whether monoclonal antibodies recognize the same antigenic site and application to hybridomes specific for the beta 2-subunit of *Escherichia coli* tryptophan synthase. *J. Immunol. Methods*, 60(3):351-358.
Gan L., Zhang Y.P. and G.X. Xiao, 1999. Production and identification of monoclonal antibodies against *Bacteroids fragilis*. *Acta Academiae Medicinae Militaris Tertiae*, 21:485-487.
Ji L.L., Wang K.Y., Xiao D. and T. Yan, 2008. Isolation and identification of pathogenic bacteria causing ulcer disease of *silurus meridionalis Chen*. *Freshw. Fish.*, 38(3):68-72 (in Chinese).
Kaper J.B., Lockman H., Colwell R.R. and S.W. Joseph, 1981. *Aeromonas hydrophila*: ecology and toxigenicity of isolates from an estuary. *J. Appl. Bacteriol.*, 50(2):359-377.
Ko W.C., Lee H.C., Chuang Y.C., Liu C.C. and J.J. Wu, 2000. Clinical features and therapeutic implications of 104 episodes of monomicrobial *Aeromonas* bacteraemia. *J. Infect.*, 40(3):267-273.
Kohler G. and C. Milstein, 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256:495-497.
Littlefield J.W., 1964. Selection of hybrids from matings of fibroblasts *in vitro* and their presumed recombinations. *Science*, 145:709-710.
Nielsen M.E., Høi L., Schmidt A.S., Qian D., Shimada T., Shen J.Y. and J.L. Larsen, 2001. Is *Aeromonas hydrophila* the dominant motile *Aeromonas* species that causes disease outbreaks in aquaculture production in the Zhejiang Province of China? *Dis. Aquat. Org.*, 46:23-29.
Situ Z.Q. and J.Z. Wu, 1996. *Cell Culture*. World Book Press, Xi’an, China.

Son R., Rusul G., Sahilah A.M., Zainuri A., Raha A.R. and I. Salmah, 1997. Antibiotic resistance and plasmid profile of *Aeromonas hydrophila* isolates from cultured fish, Telapia (*Telapia mossambica*). *Lett. Appl. Microbiol.,* 24(6):479-482.

Su J., Zhang Y.L., Yu C.X., Che X.Y. and X.N. Wang, 2002. The preparation and identification of anti-human NGF monoclonal antibody. *Chin. J. Cell Mol. Immunol.*, 18(2):168-170 (in Chinese).

Teira R., Hernandez J.L., Rojo P., Zubero Z., Munoz J. and J.M. Santamaria, 1991. Bacteremia of biliary origin caused by *Aeromonas caviae* in a healthy patient. *Enferm. Infecc. Microbiol. Clin.*, 9:388-389 (in Spanish).

Trust T.J. and R.A.H. Sparrow, 1974. The bacterial flora in the alimentary tract of freshwater salmonid fishes. *Can. J. Microbiol.,* 20(9):1219-1228.

Van der Gaag E.J., Roelofsen E. and R.F. Tummers, 2005. *Aeromonas caviae* infection mimicking inflammatory bowel disease in a child. *Ned Tijdschr Geneeskd.,* 149(13):712-714 (in Dutch).

Wang J.Z. and M. Fan, 2002. *Manuals of Protein Technical.* Sci. Press, Beijing, China.

Wang L.F. and M. Yu, 2004. Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. *Curr. Drug Targets*, 5(1):1-15.

Zhang Q.P., Wang J. and S.W. Liu, 2002. *Experimental Technique of Medical Immunology*. Wuhan Univ. Press, Wuhan, China.