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Full Paper

Identification of *Aeromonas hydrophila* infection with specific monoclonal antibodies

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Abstract: Whole cell of Aeromonas hydrophila 1234 was used for immunization to produce monoclonal antibodies (MAbs). Three different groups of MAbs specific to Aeromonas were obtained. The first group of MAbs demonstrated high specificity and bound to the A. hydrophila 1234 only but did not bind to the other two A. hydrophila isolates. This group of MAbs bound to a series of lipo-polysaccharides (LPS) with molecular masses range from 10 to190 kDa. The second group of antibodies recognized *A. hydrophila* 1234 and 2798 isolates, and bound to a series of LPS with molecular masses range from 5-200 kDa. The third group of MAbs recognized all three isolates of *A. hydrophila* and two isolates of *A. sobria*, and lightly bound to *A. caviae*. This group of MAbs also bound to an unknown protein with molecular mass of 20 kDa. The MAbs in group 1 and group 2 can be used to detect the bacteria in tissues by immunohistochemistry. Both groups of MAbs bound to LPS at different sites in which the MAbs in group 2 bound to the side chain of O antigen while

the MAbs in group 1 bound to the polymerization site at the core of oligosaccharide. All of the MAbs can be used to identify *Aeromonas* by dot blotting with the sensitivity range from 10^3-10^4 CFU/μl. This study demonstrated a convenient immunological tool that can be used for simple and accurate identification of *A. hydrophila*, as well as for diagnosis of the *A. hydrophila* infection in animals. This immunological tool can replace costly and laborious biochemical tests.

Keywords: *Aeromonas hydrophila*, dot blotting, immunohistochemistry, monoclonal antibody, lipopolysaccharide (LPS), Western blotting.

Introduction

Aeromonas hydrophila , a Gram negative motile rod, a member of the family Vibrionaceae, has been widely studied and regarded as the most important bacteria for causing "aeromonosis, or hemorrhagic septicemia or motile aeromonas septicemia" in fish]1,2,3] and other aquatic animals such as frog [4,5], prawn [6], crab [7] and mussel [8] (Maki et al., 1998). The pathological conditions include tail/fin rot and hemorrhagic septicemias in freshwater fish species and occasionally in marine fish [1,9]. The motile aeromonads has classically been divided into three biochemically different groups, namely *A. hydrophila*, *A. caviae* and *A. sobria*. These also contain at least 13 mesophilic genomospecies, and also a psychrophillic group of nonmotile *A. salmonicida* which causes disease among salmonids called "furunculosis" [10]. During the past decade the interest in *Aeromonas* spp. has gone beyond the boundaries of fish pathology due to the increasing reports of acute diarrhea in humans caused by *Aeromonas* spp. [11,12]. Up to date *A. hydrophila* has been well established as a food borne pathogen and has been isolated from both animal and plant food products [13].

Epidemiological investigation has been hampered by either the lack of rapid identification method, or taxonomic complexicities of various isolates. The conventional methods necessary to identify this pathogen are laborious, time consuming and uninventive. Recently immunoassays and molecular methods using DNA probes or PCR have been proven useful for direct detection of microorganisms present in clinical samples [2, 14]. However, most of the molecular methods for diagnosing the bacterial agents can only be effectively used by well-trained personnel and in a well-equipped setting. This limits their use in laboratories. Development of efficient (specific and sensitive), simple-to-use and rapid diagnostic methods, such as immunoassays based on monoclonal antibody (MAb) , are in general essential for detecting etiologic agents and various stages of the disease. Also, specific polyclonal antibody (PAb) against *A. hydrophila* can be used either for diagnosis [15-18, or for studying the role of flagella on bacterial invasion [19]. However, PAb could give a false positive result and an unspecific background of antigen-antibody reaction, especially in the characterization of epitopes of the target antigens. By contrast, MAb specific to *A. hydrophila* has been characterized against LPS of *A. hydrophila* type I isolates [20], and also another MAb against a 110 kDa protein of *A. hydrophila* with low cross reactivity to other *Aeromonas* spp. and various bacterial species [21]. In

this study, we demonstrated a series of MAbs specific to *A. hydrophila* isolates and *Aeromonas* spp. in order to use them as a tool for immunodiagnosis of *Aeromonas* infection.

Materials and Methods

Bacterial culture and antigen preparation

Aeromonas hydrophila 1234 (AH1) isolated from carp's kidney was kindly provided by Veterinary Medical Aquatic Research Center (VMARC), Chulalongkorn University, Thailand. *A. hydrophila* 04082 (AH2) was obtained from Aquatic Animal Health Research Institute, Department of Fisheries Thailand. *A. hydrophila* 2798 (AH3) was obtained from Department of Medical Sciences, Ministry of Public Health, Thailand. Other sources of other bacteria used for cross-reactivity testing were indicated in Table 1. The bacteria were grown with agitation at 37^{o}C in a 250 ml flask containing tryptic soy broth (TSB; Merck) to log phase. The TSB supplemented with 2% (w/v) NaCl was used for *Vibrio parahaemolyticus*. The culture was harvested by centrifugation at 3,500 X *g* for 20 min at 4 ^{o}C. Bacterial pellets were washed twice with sterile 0.15 M phosphate buffered saline (PBS) pH7.2, suspended in PBS, heat-killed at 60 ^{o}C for 30 min, and finally adjusted to the O.D. of 1 at 600 nm (approximately 10^{9} CFU/ml). The bacterial suspension was divided into aliquots and stored at -70^{o}C until used.

Three preparations (heat-killed, denatured and formalin-fixed) of *A. hydrophila* 1234 (AH1) were used for immunization. The denatured antigen was prepared by mixing the bacteria with a treatment buffer containing an equal volume of 4% sodium dodecyl sulfate (SDS) and 10% mercaptoethanol, boiling for 1 min, and then dialyzing against an excess volume of PBS three times at 12 h interval. Formalin fixed antigen was prepared by mixing the bacteria with 20% formalin at a ratio of 1:1 (v/v) for 2 h and dialyzing as before.

Immunization

The mixture of 3 preparations of *A. hydrophila* 1234 was prepared at a ratio 1:1:1. Four 6-week old female Swiss mice purchased from National Laboratory Animal Center, Mahidol University, were injected intraperitoneally with 50 μl of a prepared mixture (10^{8} CFU/ml) of *A. hydrophila* 1234 mixed with an equal volume of complete Freund's adjuvant. They were subsequently injected 3 more times with the same inoculum mixed with incomplete Freund's adjuvant at two-week intervals. One week after the fourth injection, mouse antisera were collected by eyebleeding and preabsorbed with an excess number of *V. parahaemolyticus* cells for partial elimination of the antibodies that can recognize common epitopes of both *Vibrio* and *Aeromonas*. The antisera from four mice were tested against *A. hydrophila* 1234 by Western blotting. After the best performing mouse was identified, it was boosted with the same *A. hydrophila* preparation for 3 days before hybridoma production.

Table 1. List of bacterial isolates and sources used in this study.

Bacteria	**Sources**	**Remarks**
Aeromonas hydrophila 1234	VMARC	Isolated form carp kidney
A. hydrophila 04082	AAHRI	
A. hydrophila 2798	DMST	Isolated from stool
A. sobria 12056	NCIMB	
A. sobria 12446	DMST	Isolated from stool
A. caviae 13016	NCIMB	
Plesiomonas shigelloides	DMST	Isolated from rectal swab
Vibrio alginolyticus 22082	DMST	Isolated from stool
V. cholerae Non O1 non O139	SWU	Isolated from *Penaeus vannamei*
V. fluvialis 22085	DMST	Isolated from stool
V. harveyi 639	CENTEX	Isolated from *P. monodon*
V. mimicus 22088	DMST	Isolated from food
V. parahaemolyticus 22091	DMST	Isolated from stool
V. vulnificus	DABU	Isolated from sea bass
Enterobacter cloacae	DMSM	
Escherichia coli ATCC 25922	CPF	
Klebsiella pneumoniae	DMSM	
Morganella morganii	DMSM	
Proteus vulgaris	DMSM	
Pseudomonas auroginosa	DMSM	
Salmonella Enteritidis 7108	DMST	
Salmonella Typhi	DMSM	
Salmonella Typhimurium ATCC 1408	CPF	
Shigella flexneri	DMSM	

Notes:
AAHRI = Aquatic Animal Health Research Institute, Dept. of Fisheries, Ministry of Agriculture
CENTEX = Centex Shrimp, Faculty of Science, Mahidol University
CPF = Charoen Pokphand Foods Public Co. Ltd.
DABU = Dept.of Aquatic Science, Burapa University
DMSM = Dept. of Microbiology, Faculty of Science, Mahidol University
DMST = Dept. of Medical Science, Ministry of Public Health Thailand
NCIMB = National Collection of Industrial Marine and Food Bacteria, UK
SWU = Dept. of Biology, Srinakharinwirot University
VMARC = Veterinary Medical Aquatic Research Center, Chulalongkorn University

Hybridoma production

A cell fusion protocol used in this study was modified according to the methods developed by Köhler and Milestein [22] and Mosmann et al. [23]. A P3X myeloma cell line was used as the fusion partner. Fusion products from 1 mouse were plated on 30 microculture plates (96 wells/plate). After identification of the positive cultures by screening methods including dot blotting, Western blotting

and immunohistochemistry as described below, the cells were cloned by the limiting dilution method, and stored in liquid nitrogen.

Dot-blotting

Heat-killed *A. hydrophila* and other bacterial preparations containing approximately 10^8 CFU/ml of various bacteria were used for screening. Bacterial samples (1 µl/spot) were spotted onto nitrocellulose membrane, baked at 60^{o}C for 10 min, and incubated in each conditioned medium from hybridoma culture at 1:200 dilution in 5% Blotto (5% nonfat drymilk, 0.1% Triton X-100 in PBS) for 5 h. After extensive washing in 0.5% Blotto, the membrane was incubated in horseradish peroxidase labeled with goat anti-mouse gamma immunoglobulin heavy and light-chain specific antibody (GAM-HRP, Bio-Rad) at 1:1500 dilution for 3 h. The membrane was then washed as before in Blotto and incubated for 5 min in substrate mixture containing 0.03% diaminobenzidine (DAB), 0.006% hydrogen peroxide, and 0.05% cobalt chloride in PBS [24]. Hybridoma cultures that displayed immunoreactivity against *A. hydrophila* were confirmed for bacterial specificity by Western blotting and immunohistochemistry before cloning and cryopreservation for further investigation.

SDS-PAGE and Western blotting analysis

Heat-killed *A. hydrophila* and other bacterial preparations were separated by 15% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli [25]. Samples were electrophoresed for 6 h at 30 V and gels were stained with Coomassie Brilliant Blue R-250. For Western blotting, samples resolved by SDS-PAGE were electro-blotted onto nitrocellulose membrane using Transblot apparatus (Bio-Rad). The nitrocellulose membrane was incubated in 5% Blotto for 10 min, treated with 1:200 hybridoma conditioned medium for 5 h, and then performed as described above in the dot blotting section. Low molecular weight markers (Bio-Rad) were used as a standard.

Immunohistochemistry

Ten Nile tilapia, *Oreochromis niloticus,* 1-2 g weight (purchased from Jatujak fish market, Bangkok) was artificially infected with *A. hydrophila* 1234 (50 µl of 10^7 CFU/ml) by intraperitoneal injection. When the fish exhibited pathogenic symptoms such as external ulcer, lethargy and swollen abdomen, they were killed in the cold water and fixed in Davidson fixative for 24 h and processed for paraffin sectioning.

Serial sections (8 µm thickness) were prepared and processed for indirect immunoperoxidase staining using various MAbs and GAM-HRP diluted 1:1000 with 10% calf serum in PBS. Peroxidase activity was revealed by incubation with 0.03% DAB and 0.006% hydrogen peroxide in PBS. Preparations were counter-stained with haematoxylin and eosin Y (H&E), dehydrated in graded

ethanol series, cleared in xylene and mounted in permount [24]. Positive immunoreactivity was visualized as brown coloration against the pink and purple colors of H&E.

Class and subclass determination.

Class and subclass of mouse immunoglobulins produced by the hybridomas were determined by sandwich ELISA using Zymed's Mouse MonoAb ID Kit (HRP).

Sensitivity of MAb for detection of A. hydrophila determined by dot-blotting.

Ten-fold serial dilution of *A. hydrophila* (begining with 10^8CFU/ml) in PBS was performed and 1 µl of each dilution was spotted onto a nitrocellulose membrane before fixing in 10% formalin for 10 min and processing for dot blotting using various MAbs as described above. The lowest bacterial dilution that showed distinct and clear immunoreactivity was determined.

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Results and Discussion

After the fourth immunization, the antisera from four mice at dilution of 1:20,000 were preabsorbed with the lysate of *Vibrio parahaemolyticus* before determination of the specificity by Western blotting. All antisera demonstrated a series of numerous bands without cross-reactivity to *V. parahaemolyticus* and *Escherichia coli*. The serum from mouse number 2 demonstrated the strongest immunoreactivity, therefore, it was used as spleen donor for hybridoma production.

From one fusion, the cell mixture were laid in 30 microculture plates and about half (1500 wells) contained hybridoma colonies from which approximately 200 wells gave positive reaction in the first screening by dot blotting against *A. hydrophila* 1234. After the second screening by Western blotting, antibody-producing clones with strong positive immunoreactivity and high specificity and some limited cross-reactivity to related bacteria were selected and recloned. Only 10 hybridoma clones were selected, cloned and established as high stability cell lines which can be divided into 3 groups according to their specificities (Table 2, Fig.1, 2, 3).

MAbs in group 1 consisted of 4 MAbs (AH32, 63, 66, 76) and all of them were IgG1. These MAbs bound to only one isolate of the *A. hydrophila* (1234) and did not show any cross-reactivity to the other two isolates of *A. hydrophila* (04082 and 2798) and other bacterial species (Fig.1A). The MAbs bound to a series of lipopolysaccharides (LPS) ranging from 10 to 200 kDa with increasing step of 5 kDa (Fig.2B). These MAbs can be used to detect *A. hydrophila* infection in the infected Nile tilapia by immunohistochemistry (Fig 3).

Table 2. Specificity of monoclonal antibodies

Group	MAb (isotype)	Sensitivity Dot Blotting (CFU/ml)	Antigen Western blotting (kDa)	IHC	Bacterial immunoreactivity (Dot blotting)
1	AH 32,63, 66, 76 (G1)	10^6	10-200	+++	AH1(+++)
2	AH 29 (G2a), 59 (G2b)	10^6	5-200	+++	AH1, AH3 (+++)
3	AH 17, 53 (G1), 56, 65 (G2b)	10^7	20	-	AH1, AH2, AH3, AS1, AS2 (+++) AC (+)

Notes: The binding of antibodies to various bacteria was determined by dot blotting using bacteria at approximately 10^8 CFU/ml: AH1 = *A. hydrophila* 1234, AH2 = *A. hydrophila* 04082, *A. hydrophila* 2798, AS1 = *A. sobria* 12056, AS2 = *A. sobria* 12446, AC = *A. caviae*13016. The intensity of staining was arbitrarily scored as +++ = very intense staining, + = light staining, $\pm$ very light staining - = not staining. IHC = immunohistochemistry

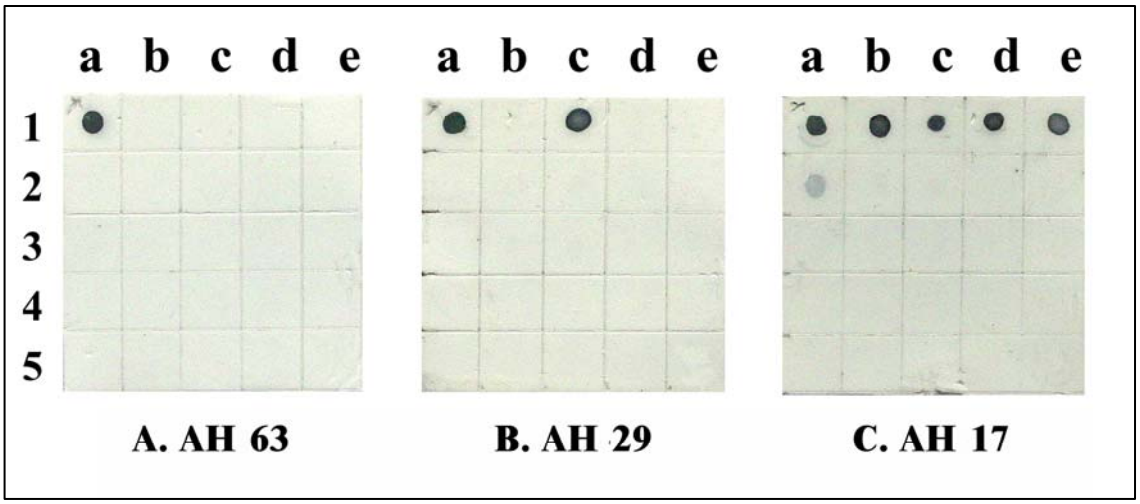

Figure 1. Cross-reactivity of MAbs assayed by dot blotting.
Heat killed bacteria (10^9 CFU/ml) were spotted on nitrocellulose membrane (1µl/spot) and treated with various MAbs: (A) AH63, (B) AH29, (C) AH17 (only one of representative for each group was demonstrated).
Row 1. a. *A. hydrophila* 1234, b. *A. hydrophila* 04082, c. *A. hydrophila* 2798, d. *A. sobria* 12446, e. *A. sobria* 12056.
Row 2. a. *A. caviae* 13016, b. *Vibrio penaeicida*, c. *V. vulnificus*, d. *V. parahaemolyticus*, e. *V. mimicus*.
Row 3. a. *V. harveyi*, b. *V. fluvialis*, c. *V. cholerae*, d. *V. alginolyticus*, e. *Pseudomonas auroginosa*.
Row 4. a. *Klebsiella pneumoniae*, b. *Proteus vulgaris*, c. *Plesiomonas shigelloides*, d. *Escherichia coli*, e. *Salmonella* Typhi.
Row 5. a. *Shigella flexneri*, b. *Morganella morganii*, c. *Enterobacter cloacae*, d. *Salmonella* Enteriditis, e. *Salmonella* Typhimurium.

MAbs in group 2 consisted of two MAbs (AH29 and AH59) having two different subclasses (IgG2a and IgG2b). They bound to *A. hydrophila* (1234 and 2798 isolates) but did not bind to *A. hydrophila* 04082 and other bacterial species (Fig.1B). The antibodies recognized a series of LPS ranging from 5 to 200 kDa with increasing step of 5 kDa (Fig.2C). This evidence suggested that the MAbs in the second group bound to epitope at the side chain of O antigen of LPS while the antibodies in the first group bound to the epitope at the core oligosaccharide on polymerization site of LPS. The antibodies in the second group also recognized the bacteria in the infected tissues similar to that of the MAbs in the first group (Fig.3-3).

MAbs in group 3 consisted of four MAbs: AH17, 53 (IgG1), 56, 65 (IgG2b). The MAbs recognized all three isolates of *A. hydrophila* and demonstrated cross-reactivity to two isolates of *A. sobria*, and one isolate of *A. caviae* (Fig. 1C). However, the immunoreactivity against *A. caviae* was weaker than the immunoreactivity to *A. hydrophila* and *A. sobria*. (Fig. 1C). These antibodies did not recognize the bacteria in the tissues by immunohistochemistry. Nevertheless, these MAbs demonstrated broad specificity against all *Aeromonas* spp. tested, the range of specificity being narrower than that of previously reported MAbs. The MAbCX9/15 was generated against *E. coli* and recognized all members of bacteria in the family *Enterobacteriaceae* with exception of *Erwinia chrysanthemi* [26]. Therefore, the third group of antibodies can be generally used for identification *Aeromonas* spp. In order to improve the sensitivity to all three *Aeromonas* spp. the additional MAb specific to *A. caviae* is required.

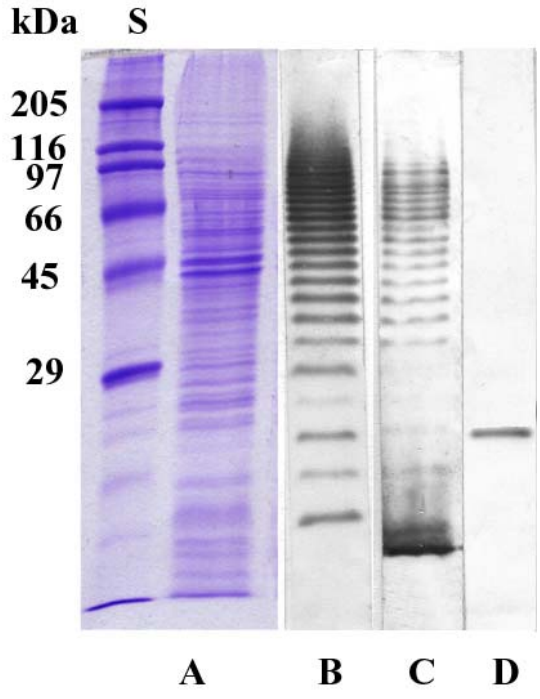

Figure 2. SDS-PAGE and Western blotting analysis.
Heat-killed *A. hydrophila* homogenate was separated by SDS-PAGE and (A) stained with Coomassie Blue. Proteins in another part of the gel was transferred to nitrocellulose membrane and treated with MAbs from each group, (B) AH63, (C) AH29, (D) AH17. S = standard marker proteins.

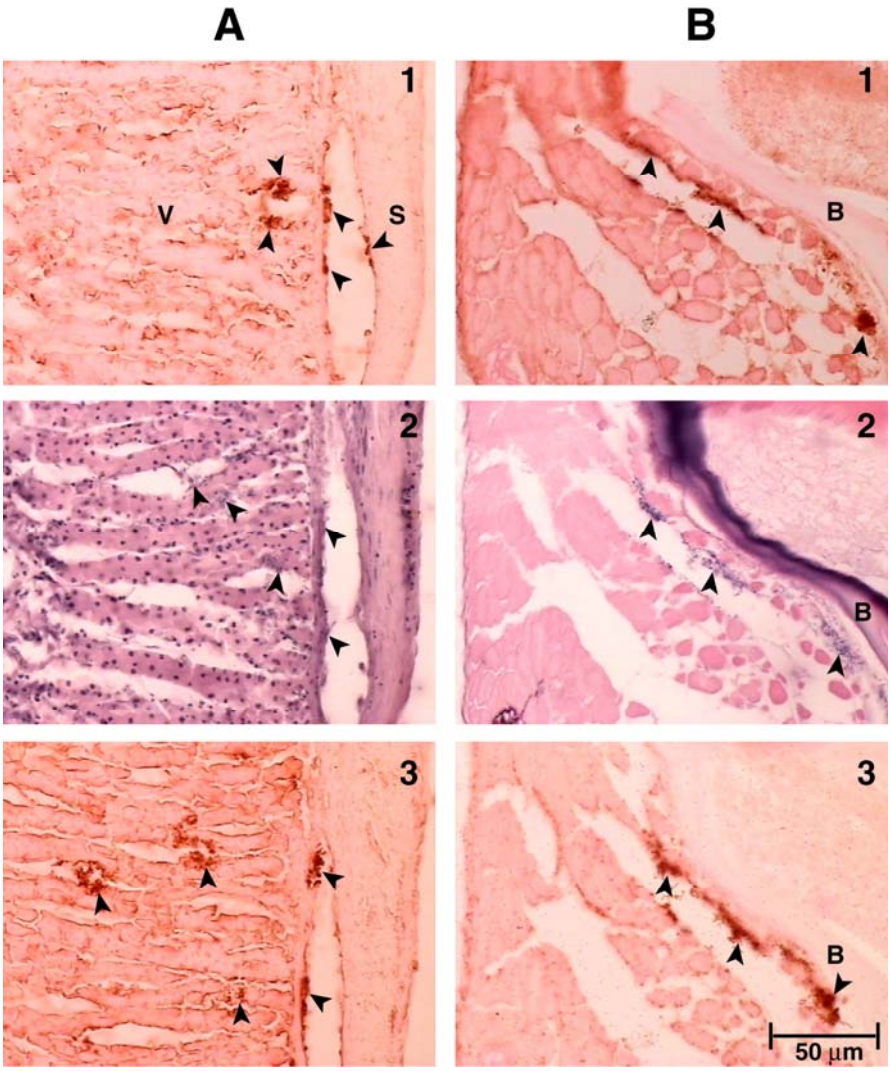

Figure 3. Immunohistochemistry.
Indirect immunoperoxidase staining of (A) intestine, and (B) skeleton muscle from experimentally infected Nile tilapia *Oreochromis niloticus* using Mabs; (1) AH63, and (3) AH29 then counterstained with eosin Y, or (2) staining with H & E without first antibody treatment. Arrow heads indicate the areas with heavy infection which were hardly observed with regular staining. B = rib bone, S = smooth muscle, V = villi.

The sensitivity testing for detection of *A. hydrophila* with dot blot assay were ranged from 10^6 to 10^7 CFU/ml depending on the group of MAbs (Table 2). Since the volume of dot blot assay was small (1 µl), the development of other types of enzyme immuno-assay such as sandwich ELISA would improve the sensitivity as the sample volume was increased by over a hundred fold. Pre-enrichment of the sample in TSB for 6 h before performing dot blotting would increase the detection sensitivity up to 1-10 CFU/ml. The *A. hydrophila* specific antibodies (group 1 and 2) can be used for detection of *A.*

hydrophila in the tissues of infected fish by means of immunohistochemistry. The immunoreactivity appeared at the surface of bacteria and soluble components in the intercellular space of various tissues (Fig.3). The detection of bacterial infection can be observed even in the area with light infection containing only a few bacterial cells or fragments which could not be observed with regular haematoxylin and eosin staining (Fig. 3B). The reason for the third group of antibodies that could not recognize the bacteria in the tissues may be due to the fact that the antibodies were specific to intracellular components. Therefore, after tissue fixation the high density of cell wall may prevent the accessibility of antibody into the cell.

The MAbs in the first group were highly specific to *A. hydrophila* 1234 without any cross-reactivity to the other two isolates of *A. hydrophila*. This evidence indicated the uniqueness among the heterogeneity of *A. hydrophila* in which different serotypes usually contain a unique epitope for each serotype.

The MAb specific to the common epitopes among the three *A. hydrophila* isolates was not obtained in this study. This evidence suggested that all three isolates of *A. hydrophila* used in this experiment were immunologically different. Previous report showed that MAb 5F3 recognized a protein of 110 kDa and could react with all 12 isolates of *A. hydrophila* from different sources including human stool, human wound and cold blood animals with very light reaction against *A. sobria*. [21]. The production of MAbs specific to various isolates of *A. hydrophila* is required in order to obtain MAbs that can react to most of the *A. hydrophila* isolates.

The antigen recognized by MAbs group 1 and 2 is expected to be a lipopolysaccharide (LPS) as described previously [20]. However, the difference in the lowest band of antigen recognized by group 1 and group 2 antibodies indicated that the epitope recognized by MAbs in group 2 may be on the O antigen with highly repetitive carbohydrate side chains of LPS monomer, while the epitope recognized by MAbs in group 1 may be on the polymerization site at the core of oligosaccharide. Therefore, the antibodies in group 1 could not recognize the smallest monomer of 5 kDa band. A MAb specific to LPS of *A. hydrophila* (F26P5C8) from previous report recognized many virulent and avirulent isolates of *A. hydrophila* isolated from diseased and healthy fish from Malaysia and Philippines. However, this antibody recognized only some isolates from diseased fish from Japan [20]. This evidence indicated that there are heterogeneity among *A. hydrophila* LPS recognized by the MAb. An experiment on production of rabbit antisera against various serotypes of motile *Aeromonas* revealed that various species of *Aeromonas* are different not only in LPS components but also in extracellular products among various serotypes and in the member of the same serotype as well [27]. Similar report on production of MAb against LPS of *A. salmonicida* demonstrated that three MAbs against core-oligosaccharide recognized all 10 typical isolates, 5 atypical isolates of *A. salmonicida*, and cross-reacted to two isolates of *A. hydrophila*, while three other MAbs recognized only *A. salmonicida* [28]. This evidence indicated that specific and common epitopes on LPS are present among various *Aeromonas* spp. and possibly can be recognized by the antibody. In our case, MAbs specific to *A. hydrophila* 1234 were generated; therefore, more production of monoclonal antibodies against different isolates of *A. hydrophila* is needed in order to provide immunological tools for identification of various isolates of *A. hydrophila*.

Conclusion

This study demonstrated that MAbs can be used for simple and accurate identification of *Aeromonas* spp. and some pathogenic isolates of *A. hydrophila* by dot blotting and immunohistochemistry. This immunological tool can replace costly and laborious biochemical tests and molecular identification. However, production of more MAbs specific to other isolates of *A. hydrophila* is required.

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