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A Detection Kit for *Aeromonas hydrophila* Using Antibody Sensitized Latex

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Abstract *Aeromonas hydrophila* is a pathogen to fish as well as human. It is a food-borne disease, and causes severe mortality in fish, and sometimes severe septicemia in human. In this study, a rapid detection method using latex agglutination has been developed for *A. hydrophila*. Polyclonal antibodies were raised against membrane and whole cells of three isolates from rainbow trout. Among these, latex particles coated with antibodies raised against whole cells of isolate No. 2 showed the best sensitivity. With latex particles coated with this antibody, we could detect 5×10^4 CFU of *A. hydrophila* in 5 min. The cross-reactivity with bacteria constituting the normal intestinal microflora and other pathogens for rainbow trout was insignificant. This latex agglutination assay method produced positive reaction with all clinical isolates of *A. hydrophila* which were identified by species-specific PCR for 16S rRNA in *A. hydrophila*.

Key words: *Aeromonas hydrophila*, agglutination, antibody, detection kit, latex agglutination

Aeromonas spp. comprise mesophilic motile and psychrophilic nonmotile, Gram-negative, ubiquitous bacteria and have been recognized as a significant opportunistic pathogen for both human and fish [3, 14, 25, 31]. World-wide studies have demonstrated that *Aeromonas* spp. are universally distributed and widely isolated from clinical, environmental, and food samples, where they may grow even at low temperatures [10]. *Aeromonas* spp. are opportunistic pathogens that are infectious and enterotoxigenic [31]. Routine detection of pathogenic *Aeromonas* spp. was not efficient until now, since they have diversed hybridization groups (HGs) and also 17% of the isolates could not be grouped into any of the known HGs with biochemical tests [7]. Thus, numerous amount of efforts were dedicated to develop a rapid detection method which can be easily used in fish farms, food industries, and especially in a clinical environment. Over the past several years, a significant number of immunochemical and DNA-based techniques have been introduced into the field of bacterial identification and taxonomy [1, 4, 5, 12, 15, 20, 22, 23, 26, 29, 33, 34]. The development of immunological techniques resulted in antibody capture, enzyme linked immunosorbent assay [16, 19], and latex agglutination test [6, 27, 30, 32]. Recently, a fluorescent antibody technique has been used with success for the identification of bacteria [9, 13].

The prerequisite of these immunochemical methods to be applied to *A. hydrophila* is the specificity without cross-reaction with other aeromonads or non-*Aeromonas* organism, because of the wide phenotypic variation of *A. hydrophila*. Accurate, reproducible, and rapid results obtained by latex agglutination to screen *Candida albican* [28], *Aeromonas salmonicida* [24], and various toxins [21, 33] encouraged us to develop a similar detection method for *A. hydrophila*. Uniform latex particle was first discovered in 1947. Since then, they have been applied widely to electron microscopy, cell counter calibration, antibody mediated agglutination diagnosis, phagocytosis experiments, and many others.

In this study, we developed the first latex agglutination method for *A. hydrophila* with insignificant cross-reactivity with other bacteria constituting normal intestinal microflora and pathogens of rainbow trout, and evaluated its specificity and efficiency with clinical isolates.

Materials and Methods

Bacterial Strains

Bacteria constituting normal intestinal microflora (*Enterobacteriaceae*, *Moraxella*, *Alteromonas*, *Flavobacterium*, and *Pseudomonas*) were isolated from rainbow trout and identified with various biochemical/physiological methods according to Bergey's Manual [17]. Eight strains of *A.*

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hydrophila, which were isolated from rainbow trout and identified in a previous work [18], were used in this study. The American type strain of *A. hydrophila* was provided by Dr. Scott LaPatra (Clear Springs Foods Inc., U.S.A.). Trout pathogens were obtained from various sources: *Vibrio anguillarum* from Prof. K. J. Jung (Seoul National University); *A. salmonicida* from Institute of Microbiology at Seoul National University (IMSNU); *Yersinia ruckery* and *Cytophaga johnsonae* from Dr. LaPatra. *Aeromonas* spp. isolated from fish were from Culture Collection of Antibiotic Resistant Microbes. Clinical isolates of *A. hydrophila* were obtained from patients in Severance Hospital, Yonsei University.

Antibody Preparation

Bacterial cells of Nos. 1, 2, and 4 were cultured in Brain Heart Infusion (BHI) broth overnight and collected by centrifugation at 8,000 ×g for 10 min at 4°C. Bacterial cells were washed three times with phosphate buffered saline (PBS) with centrifugation, suspended in PBS and inactivated by heating at 60 for 1 h after treatment with formalin (final 0.3%) for 3 h. Membrane parts were prepared as follows: the bacterial cell pellet was suspended in PBS (pH 8.0) and broken in a French pressurized cell (SLM-AMINCO, Rochester, NY, U.S.A.). Unbroken cells were removed by centrifugation at 8,000 ×g for 10 min. The supernatant was centrifuged at 100,000 ×g for 1 h and the pellet containing the membrane parts was suspended in PBS and used as an antigen. Rabbits (New Zealand, white species, male) were intramuscularly injected with antigen (1 mg/kg weight of rabbit) mixed with Freund's incomplete adjuvant (FIA) (Difco, U.S.A.) for three boosts, once intramuscularly and twice subcutaneously. Antibody formation was assayed by enzyme linked immunosorbent assay (ELISA). Blood was sampled 2 weeks after the last immunization and allowed to clot at 37°C. Serum was prepared by centrifugation and stored at -20°C. Sera were obtained from the rabbits prior to injection and used as the control serum. IgG was purified from serum using a protein A column following a procedure described elsewhere [11].

Latex Sensitization

Latex sensitization was performed following the procedure described elsewhere [2]. Latex particles suspended in 0.17 M glycine buffer containing 0.1% TX-100 were mixed with 250 mg IgG. Sensitized latex particles were washed with the same buffer by centrifugation. To find out the optimum concentration for the detection, IgG was serially diluted in glycine/NaOH (pH 8.2). The concentration which produced the strongest agglutination was considered as the optimum concentration for each IgG.

Agglutination Test

Half of each single colony was picked and suspended in PBS. The turbidity was adjusted to 0.5 MacFarland unit. An aliquot (5 µl) of the suspended cells and 5 µl of the sensitized latex particles were dropped on the reading panel and gently mixed by rotating for 5 min.

PCR

Half of each bacterial colony was transferred to a PCR tube. Cells were broken with microwave at 700 watts for 104 s. A PCR reaction mixture (50 µl) consisted of 1.25 U of *Taq* DNA polymerase (Takara Shuzo Co., Ltd., Japan), 5 µl of 10× PCR amplification buffer [100 mM Tris-Cl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 2 mM dNTP mixture], and 50 pmole of each primer. Primers targeted to 16S rRNA genes were as designed by Dorsch *et al.* [8]. For the amplification, *Taq* DNA polymerase was added to the template after the initial denaturation at 98°C for 150 s. The PCR was constituted with 5 cycles of denaturation at 93°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 30 s and 28 cycles of denaturation at 93°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s. The final extension was performed at 72°C for 30 min following annealing at 60°C. Each set of reactions included a negative control to rule out contamination from buffers, enzyme, and primer solutions.

PCR products were visualized with ethidium bromide after electrophoresis on a 1% agarose gel.

Results and Discussion

Optimum Concentration of Antibody to Label Latex Particles

The optimum concentration of each antibody for latex binding ranged from 0.0156 to 10 µg/µl. Antibodies raised against total proteins of No. 2 were selected for further experiments based on their lowest optimum concentration, 0.0156 µg/µl.

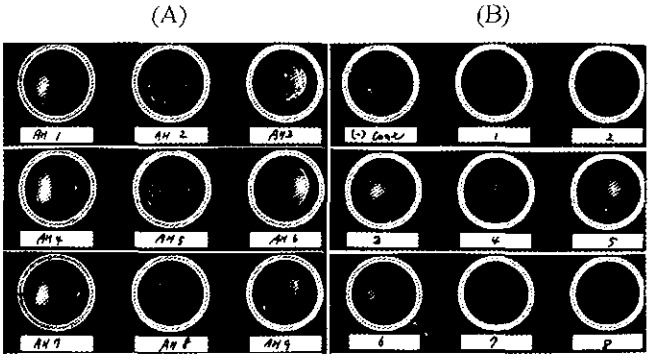

Fig. 1. Latex agglutination test.
Latex particles sensitized with antibodies were mixed with isolates of *A. hydrophila* which were identified with species-specific PCR (A) and bacteria constituting the normal intestinal microflora in rainbow trout and fish pathogens (B). (A) Isolates of *A. hydrophila*. (B) 1, *Alteromonas*; 2, *Enterobacteriaceae*; 3, *Flavobacterium*; 4, *Moraxella*; 5, *Pseudomonas*; 6, *A. salmonicida*; 7, *Cytophaga johnsonae*; 8, *Vibrio anguillarum*.

Table 1. Latex agglutination test of *Aeromonas* spp.

Aeromonas spp. isolate No.	Agglutination
1	-
2	-
3	-
4	-
5	-
6	-
7	-
8	-
9	-
10	-
11	+
12	-
Without bacterial cells	-
Aeromonas hydrophila	+

Optimum Concentration of TX-100 to Prevent Nonspecific Binding

False positive agglutination in the absence of bacteria and nonpecific agglutination with non-*Aeromonas* could be eliminated by increasing the concentration of Triton X-100 up to 2%. However, agglutination with *A. hydrophila* also decreased at this concentration, and the final concentration of 0.2% Triton X-100 was selected to be used and the detection limit at this concentration was 5×10^4 CFU of *A. hydrophila*. This sensitivity was similar to that reported by others for *A. salmonicida* [24], which was 1×10^4 CFU of *A. salmonicida*.

Specificity of Latex Agglutination

Latex particles coated with this antibody showed agglutination with all the isolates of *A. hydrophila* from rainbow trout (Fig. 1A), however, it did not agglutinate with bacteria which constitute normal intestinal microflora in rainbow trout or other pathogens (Fig. 1B). When bacterial cells of *Aeromonas* spp. isolated from various fishes were tested, eleven strains out of twelve showed negative results (Table 1).

Detection of Clinical Isolates of *A. hydrophila* with PCR and Latex Agglutination

Among twenty clinical isolates which were identified as *A. hydrophila* by conventional methods used in a hospital clinical microbiology laboratory, such as staining and biochemical/physiological tests, only nine isolates produced

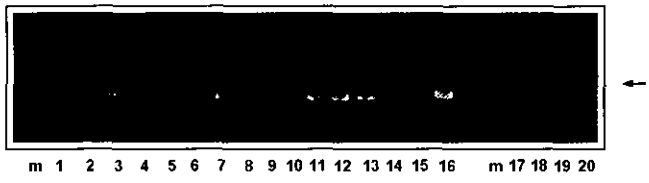

Fig. 2. PCR with primers specific to 16S rRNA of *A. hydrophila*. Genomic DNA were isolated from eighteen clinical isolates of *Aeromonas* and PCR was performed with primers specific to 16S rRNA of *A. hydrophila* as described in Materials and Methods. The arrow indicates the PCR products at 685 bp.

Table 2. Latex agglutination with clinical isolates of *A. hydrophila*.

Isolate No.	Latex agglutination	PCR of 16S rRNA
1	+	+
2	-	-
3	+	+
4	-	-
5	-	-
6	-	-
7	+	+
8	+	+
9	-	-
10	-	-
11	+	+
12	+	+
13	+	+
14	-	-
15	-	-
16	+(weekly)	+
17	-	-
18	+	-
19	+ (weekly)	+
20	-	-

PCR products with an expected size of 685 bp when performed with primers specific to 16S rRNA gene (Fig. 2). All these PCR positive isolates showed positive results with the latex agglutination method, whereas all the PCR negative isolates, except one (No. 18), showed negative results (Table 2).

Since this latex agglutination method is very easy to perform and it takes only 5 min to obtain results, we expect this method to be used in fish farms, food industries, and a clinical environment. In particular, in places without expensive equipments and experienced personnel.

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