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Research Article

Development of a co-agglutination method for detection of *Aeromonas hydrophila* as causative agent of motile *Aeromonas* septicemia (MAS) disease in gourami *(Osphronemus goramy)*

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Abstract

Aeromonas hydrophila is an opportunistic pathogen causing high mortality and economic burden in freshwater fish farming. This study aims to develop a co-agglutination method for detecting and creating *Aeromonas hydrophila* diagnostic rapidly. In this study, we injected rabbits (±2kg weight) with 1mL of *A. hydrophila* antigen suspension 1.2 x 10^9cfu mL^{-1} at one week intervals (three times, intra vena) respectively. The gouramis (15.48±0.55g-1 weight) were infected by *Aeromonas hydrophila, Aeromonas sobria, Aeromonas salmonicida, Streptococcus agalactiae,* and *Pseudomonas aeruginosa* separately with 0.1 mL $fish^{-1}$ and 10^8 cfu mL^{-1} bacterial cell suspensions. The antiserum was purified to couple with the *Staphylococcus aureus* suspension protein A, in a 1:1 (v/v) ratio and used by the co-agglutination reagent. We compared this method with standard polymerase chain reaction (PCR) for *A. hydrophila* detection. The rabbit antibody reaction occurred only against *A. hydrophila* antigen showing specificity of the gourami tissue supernatant within 10-30 seconds. The sensitivity test had a detection limit of 10^6 cfu mL^{-1}. Comparison detection method with PCR showed that positive result of *A. hydrophila* was located in 209 bp. Co-agglutination method could detect *A. hydrophila* in the internal organ of fish at 12h after injection, but the PCR method could detect at one hour after injection. This research concluded that co-agglutination method could detect *A. hydrophila* specifically, sensitively, rapidly and practically in laboratory and field examination.

Keywords: *Aeromonas hydrophila,* Diagnostic, Rapid, Co-agglutination Method.

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Introduction

Gourami (*Osphronemus goramy*) is a popular cultured species in Southeast Asia region due to high price and high nutrient value (Vidthayanon, 2012). Kusdarwati *et al.* (2018) reported *Aeromonas hydrophila* as an opportunistic pathogen capable of producing motile *Aeromonas* septicemia (MAS) in gourami. Besides gourami *A. hydrophila* could also attack other aquatic organisms, such as crayfish (*Astacus leptodactylus*), grass carp (*Ctenopharyngodon idella*), catfish (*Hemibagrus nemurus*) and zander (*Sander lucioperca*) (SamCookiyaei *et al.,* 2012, Pourgholam *et al.,* 2013, Farhana *et al.,* 2015, Faeed *et al.,* 2016).

A. hydrophila is a major influence pathogen bacteria in raising freshwater fish with a high death rate of between 80% and 100% in a relatively short time (1-2 weeks). The virulence of A. *hydrophila* which could couse the death comes from a produced toxin. Genes of aero and hlya are responsible for producing the poison aerolysin and hemolysin to genus aeromonas (Yousr *et al.,* 2007).

A. hydrophila detection using conventional methods was time consuming and need particular laboratory materials and equipments, as well as competence of personnel. This method is often applied to determine the presence of *A. hydrophila* (Kusdarwati *et al.,* 2017). In contrast polymerase chain reaction (PCR) and immunoassays (enzyme-linked immunosorbent assay (ELISA), flow immunogold assay (FIA), agar gel precipitation (AGP) and Agglutination) offer advantages but require adequate personnel competence, expensive materials and equipments, the place must be in the laboratory and not portable. Many researchers who had identified *A. hydrophila* with PCR technique and immunoassay techniques showed positive results of *A. hydrophila* detection (Amanu *et al.,* 2015a, Mufidah *et al.,* 2015, Rasmussen-Ivey *et al.,* 2016, Stratev *et al.,* 2016, Hong *et al.,* 2017, Yang *et al.,* 2017, Ballyaya *et al.,* 2018, Rakib *et al.,* 2018).

Co-agglutination is a serological test that provides specific antigen and antibody reactions within seconds. Some researchers conducted co-agglutination tests against fish or shrimp bacterial diseases, such as Evan (2017) for *Vibrio parahemolyticus*, Fikar *et al.* (2015) for *Edwardsiella ictaluri*, and Dublin (2012) for *Aeromonas salmonicida*. Worldwide outbreaks of MAS caused by *A. hydrophila* need rapid diagnostic to control this pathogen. Therefore development of a co-agglutination method is an important trend for fish bactericidal detection. The aim of the present study was to develop a co-agglutination method for detection of *A. hydrophila* antigen as a simple, rapid, specific and sensitive alternative laboratory or field diagnostic test.

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Materials and methods

Characterization and identification of bacteria

Aeromonas hydrophila, Aeromonas salmonicida, Aeromonas sobria, Pseudomonas aeruginosa, Streptococcus agalactiae, *Staphylococcus aureus* Cowan I and *Staphylococcus epidermis* used in this study were collection from Microbiology Laboratory of Fish Disease Inspection and Environment of Serang-Banten, Indonesia. *Aeromonas hydrophila* bacteria was re-identified with PCR at 209bp (Pollard *et al.*, 1990) and automatic identification tool (Sanders 2019) with 94% probability, and other bacteria were biochemically identified using vitex 2 compact for with reliable probability *A. salmonicida* (93%), *A. sobria* (99%), *S. agalctiae* (98%), *P. aeruginosa* (93%), *S. aureus* Cowan I (95%) and *S. epidermidis* (95%).

Detection of protein A in S. aureus Cowan I

Protein A in *S. aureus* Cowan I was detected using Djannatun (2016) method. In brief, *S. aureus* was grown at 37^{o}C for 24h in brain heart infusion (BHI) medium. The isolate was transferred to soft agar (SA) and serum-soft agar (SSA) medium and incubated at 37^{o}C for 24h. Rabbit serum and chicken serum were added to the SSA medium (Ningrum *et al.*, 2016).

Production of polyclonal antibody serum

A. hydrophila was inoculated on Tryptic Soy Agar (TSA) for 18-24h at 30^{o}C. Harvesting of bacteria was conducted by dissolving bacterial biomass into a physiological solution (0.85% NaCl) in a sterile tube and washed three times. Bacterial inactivation was conducted by water bath at 60^{o}C temperature for one hour followed by bacterial suspension centrifugation at 4.000rpm for 10min. Subsequently, 0.3% physiological formaldehyde solution was added as preservative.

Rabbit (2kg body weight) was injected through intravenous with 1000µL of 1.2 x 10^{9}cfu mL^{-1} *A. hydrophila* antigen. The antigen injection was carried out three times at one week intervals. The polyclonal antibody (antiserum) was harvested from rabbits three weeks after the injection**.** Measurements of antibody titers were performed at weeks 0, 2 and 3 using Tizard's (1988) method with modification. The complement of antiserum was inactivated at 56°C for 30min.

Purification of immunoglobulin G (IgG)

Purification of IgG was conducted based on Amanu *et al.* (2015b) with modification, A total of 10mL of rabbit antiserum was added to 10mL 50% ammonium sulfate with a pH of 8.0 (1:1) by dropping method for 30min, then centrifuged at 3.000rpm for 30min. The supernatant was removed and

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Phosphate buffered saline (PBS) with a pH of 7.2 was added to the resulting sediment reaching to the initial volume. Dialysis process was done using a dialysis membrane in PBS solution (pH 8.0) for 24 hours at 4ºC, PBS was changed every 8h. The resulting serum from ammonium sulfate precipitation was purified again using Melon Gel IgG Purification Kit according to the kit protocol.

Preparation of Staphylococcus aureus

Preparation of *S. aureus* was conducted according to Amanu *et al.* (2015b) with modification. In brief, *S. aureus* was cultured on TSA and incubated at 37°C for 24h. The isolate was collected in tubes containing PBS (pH 7.2) and washed three times. Formalin 0.3% was added, then incubated for 24h at room temperature. The suspension was washed and resuspended again with PBS until reaching the initial volume. The bacterial suspension was heated at 60°C for one hour and cooled directly, then centrifuged at 3.000rpm for 15min. The supernatant was removed and added with PBS up to the initial volume, and this suspension was used as the material in the test for co-agglutination.

Co-agglutination reagent producing

Co-agglutination reagent was prepared by performing ratio between purified antiserum with *S. aureus* suspension which known to have protein A that results in the absence of self agglutination. The same volume of *S. aureus* with *A. hydrophila* antiserum was incubated for 90min at 30°C. The suspension was centrifuged at 3.000rpm for 15min, the supernatant was discarded and PBS was added back to reach the initial suspension volume.

Pathogenicity test

Gourami weighing 15.48±0.55 g^{-1} was originated from Center for Freshwater Aquaculture, Curug Barang, Pandeglang Regency Banten Province, Indonesia (6º 36’ 14”S 106º 02’ 57” E) and verified by PCR testing that did not carry *A. hydrophila* Prior to injection, fish were acclimated for three days as an adaptation to avoid stress on the fish. Fish was Injected with *A. hydrophila* of 10^8cfu $fish^{-1}$ as positive control. While the negative control of fish was injected with *A. sobria, A. salmonicida, P. aeruginosa, S. agalctiae* with the same dose and PBS. The muscle, liver, and kidney of fish were used as antigens in co-agglutination test. In addition, clinical symptoms were examined and periodically those organs of each fish were tested for *A. hydrophila* antigen by co-agglutination method.

Preparation of Supernatant Antigen

Liver, kidney and muscle of *A. hydrophila, A. sobria, A. salmonicida, P. aeruginosa,* and *S. agalctiae* infected fish were crushed three to five times with PBS suspension. The organs were heated in 30min at 100°C and centrifuged at 4.000rpm for 10min. The supernatant was used as the test sample in the co-agglutination test.

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A. hydrophila was cultured on TSA for 18-24h at 30°C. Harvesting of bacteria was conducted by washing bacteria three times. Bacterial dilution was performed with an initial density of 10^9cfu mL^{-1} up to 10^1cfu mL^{-1}. Each bacterial suspension was heated at 100°C for 30min and centrifuged at 4.000rpm for 10min.

The sample test using co-agglutination reagent

Supernatant antigen was dropped on the glass object and the same volume of co-agglutination reagent was added. A total of 25µL serum and 25µL supernatant was placed in glass object and homogenized with continuous observation for 1-30sec against contrasting background.

Specificity and sensitivity test

Specificity test was conducted by adding antiserum against antigen of *A. sobria, A. salmonicida, P. aeruginosa* and *S. agalactiae* to cause negative agglutination reaction. The sensitivity characteristics tests were carried out by serial method dilution of 10^9cfu mL^{-1} up to 10^1cfu mL^{-1}. The lowest dilution was still capable of forming the antigen-antibody binding reaction which was the result of sensitive property.

Comparison with PCR (Polymerase Chain Reaction) assay

A. hydrophila, A. sobria, A. salmonicida, S. agalctiae and P. aeruginosa were extracted based on Genomic DNA Mini Kit for tissue extraction. Amplification was performed using primers forward primer (5'-CCAAGGGGTCTGTGG-CGACA-3') and reverse primer (5'-TTTCACCGGT AACAGGATTG-3', Pollard *et al.,* 1990). PCR program for DNA amplification with an initial denaturation of 95°C for 4min, denaturation of 95°C for 30sec, annealing at 54°C for 45sec and extension at 72°C for 30sec, all steps were cycled for 30 cycles. Finally, the process was finished by extension at 72°C for 10min with the final temperature of 40°C. PCR results of various treatments were electrophoresed on agarose gel. Electrophoresis was run with 100 volt voltage for 23min and was observed above the UV transilluminator.

Results

Polyclonal antibody reaction

The polyclonal antibody reaction to rabbits injected with *A. hydrophila* antigen showed agglutination reaction at weeks two and three. Specific reaction of polyclonal antibody serum to *A. hydrophila* was the presence of agglutinic particles (sand-like grains, Fig. 1). Negative reactions were shown in the negative controls which were reacted to *A. sobria, A. salmonicida, P. aeruginosa* and *S. agalactiae* with no agglutinate particle (homogeneous suspension, Fig. 2).

The cross reactions tested with *A. sobria, A. salmonicida, P. aeruginosa* and *S. agalactiae* showed no

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agglutination reaction. The method performed in the cross reaction test showed that serum polyclonal antibodies were specific only to the whole cell of *A. hydrophila* (Tabel 1). Results of antibody titer measurement showed that there was an increase of antibody titer value from day 14 at 1:32 to day 21 at 1:128 (Table 2).

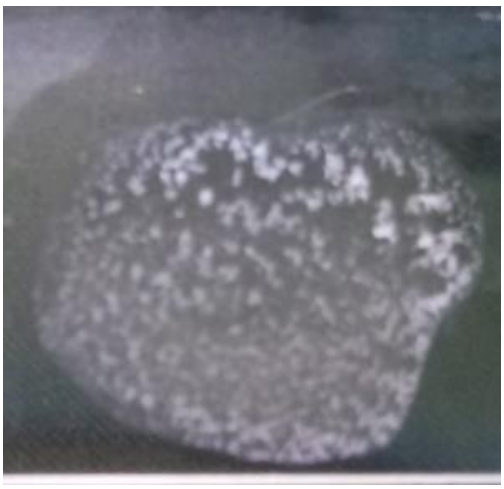


Figure 1: Positive agglutination reaction base on polyclonal antibody.



Figure 2: Negative agglutination reaction base on polyclonal antibody.

Detection of protein A in S. aureus Cowan I

The rabbit serum added with *S. aureus* Cowan I on SSA showed bacteria with compact colonies, whereas in SA testing medium showed diffuse colonies (Fig. 3). The difference of results from each medium assay is provided in Table 3. Compact and defuse colonies of *S. aureus* were present in added rabbit serum and chicken serum, also diffuse colonies of *S. epidermis* were present in SA, rabbit serum or chicken serum.

Table 1: Determination of cross reaction of *A. hydrophila* polyclonal antibody, - negative, + positive.

Bacterial antigen	Agglutination
A. hydrophila	+
A. sobria	-
A. salmonicida	-
P. aeruginosa	-
S. agalactiae	-

Co-agglutination reagent assay

The results of co-agglutination test on muscle, liver and kidney of fish infected by *A. hydrophila, A. sobria, A. salmonicida, P. aeruginosa, S. agalactiae* and uninjected control are presented in Table 4. Positive reaction occurred only at fish organs injected by *A. hydrophila*. The organs of fish injected with *A. sobria, A. salmonicida, P. aeruginosa, S. agalactiae*, and uninjected control showed negative reaction.

The positive co-agglutination reaction caused by *A. hydrophila* antigen was formed within 10-30 seconds. The complex bonding process that consists of antigen antibodies which results in the size of the molecule was getting bigger so it could be seen directly getting the smooth grain like white sand (Fig. 4).

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Table 2: Antibody titer test measurements.+have agglutination, - dose have agglutination; 1 (1:1 dilution), 2 (1:2 dilution), 3 (1:4 dilution), 4 (1:8 dilution), 5 (1:16 dilution), 6 (1:32 dilution), 7 (1:64 dilution), 8 (1:128 dilution), 9 (1:256 dilution), 10 (1:512 dilution), 11 (1:1024 dilution), 12 (1:2056 dilution).

Time	Agglutination in a serum dilution											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Day 0	-	-	-	-	-	-	-	-	-	-	-	-
Days 14	+	+	+	+	+	+	-	-	-	-	-	-
Days 21	+	+	+	+	+	+	+	+	-	-	-	-



Figure 3: Compact colonies of *S. aureus* in rabbit serum SSA media and diffuse colonies in SA media.

Table 3: Shape of bacterial colonies of *Staphylococcus aureus* and *Staphylococcus epidermidis* in serum-soft agar (SSA) test and soft agar (SA) test.

Isolates	Media testing		
	Soft agar (SA)	Serum-soft agar (SSA)	
		Rabbit serum	Chicken serum
Staphylococcus aureus Cowan I	Diffuse	Compact	Diffuse
Staphylococcus epidermidis	Diffuse	Diffuse	Diffuse

Table 4: Co-agglutination test results on gourami organ sample, (+) positive reaction; (-) negative reaction.

Bacterial infections	The reaction of agglutination on organs											
	Muscle				Liver				Kidney			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Aeromonas hydrophila	+	+	+	+	+	+	+	+	+	+	+	+
Aeromonas sobria	-	-	-	-	-	-	-	-	-	-	-	-
Aeromonas salmonicida	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus agalactiae	-	-	-	-	-	-	-	-	-	-	-	-
Without infection	-	-	-	-	-	-	-	-	-	-	-	-

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Figure 4: Reaction of co-agglutination reagents: Positive agglutination reaction.

Each fish injected with *A. sobria*, *A. salmonicida*, *P. aeruginosa*, *S. agalactiae* and control without injection of bacteria showed a homogeneous and agranular reaction which indicate a negative reaction (Fig. 5).

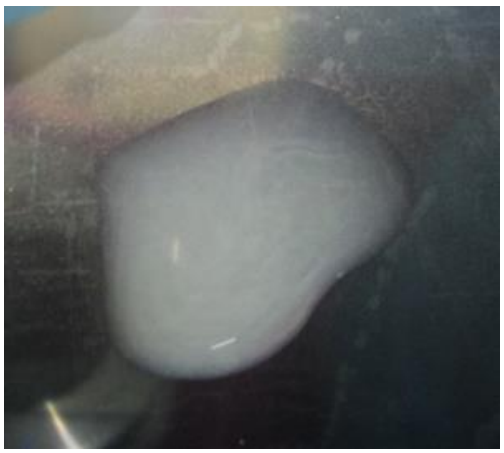


Figure 5: Reaction of co-agglutination reagents: Negative agglutination reaction.

Specificity and sensitivity of co-agglutination reagent

Specificity of co-agglutination reagent indicated that the presence of *A. hydrophila* antigen was detected by the reagent (Fig. 6). Co-agglutination reagent exhibited a 30-second test limit on *A. sobria*, *A. salmonicida*, *P. aeruginosa* and *S. Agalactiae*, and produced negative agglutination. Fragment antigen binding (Fab) in co-agglutination reagent had specific character showing *A. hydrophila* increased existence and could bind to its specific antigen (Fig. 6A).

The co-agglutination reagent isolated *A. hydrophila* at sensitivity level starting from 10^9cfu mL^{-1} up to 10^1cfu mL^{-1}. It showed that density of 10^9cfu mL^{-1} until 10^6cfu mL^{-1} detect *A. hydrophila* antigen as sensitivity limit with an indication of particle agglutination (Fig. 7A-D).

Obviously, co-agglutination reagent *A. hydrophila* antigen detection limit was 10^6cfu mL^{-1}, because the density of 10^5cfu mL^{-1} until 10^1cfu mL^{-1} showed negative reaction (Table 5).

Detection of A. hydrophila with PCR

PCR method only detected *A. hydrophila* as positive result, while negative result is shown for *A. sobria*, *A. salmonicida*, *P. aeruginosa* and *S. agalactiae* (Fig. 8). The synthetic aerolysin specific, base on oligonucleotide primers, targeting 209bp fragment of the aerolysin gene, coding for hole forming aerolysin toxin, detected *A. hydrophila*. In contrast, no similar fragment was observed in the PCR method when the template nucleid acid from *A. sobria*, *A. salmonicida*, *P. Aeruginosa* and *S. Agalactiae* was examined.

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Figure 6: Specificity of co-agglutination reagent: (A) *A. hydrophila*, (B) *A. sobria*, (C) *A. salmonicida*, (D) *P. aeruginonas*, (E) *S. agalactiae*, (D) non-infection.

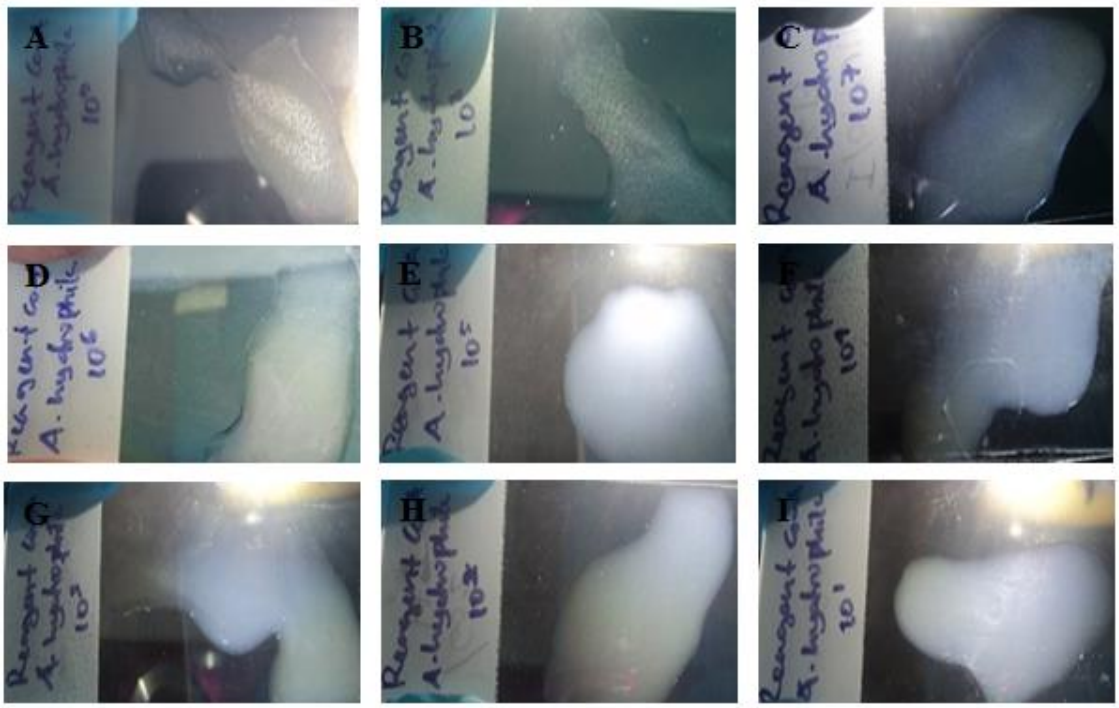


Figure 7: Sensitivity of co-agglutination reagent: (A) *A. hydrophila* 10^9cfu mL^{-1}, (B) *A. hydrophila* 10^8cfu mL^{-1}, (C) *A. hydrophila* 10^7cfu mL-1, (D) *A. hydrophila* 10^6cfu mL-1, (E) *A. hydrophila* 10^5cfu mL^{-1}, (F) A. hydrophila 10^4cfu mL^{-1}, (G) *A. hydrophila* 10^3cfu mL^{-1}, (H) *A. hydrophila* 10^2cfu mL^{-1}, (I) *A. hydrophila* 10^1cfu mL^{-1}.

Table 5: Sensitivity of co-agglutination reagent to *A. hydrophila* antigen, (+) positive reaction, (-) negative reaction.

Density of *A. hydrophila* antigen	Cfu mL^{-1}								
	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1
Co-agglutination reaction	+	+	+	+	-	-	-	-	-

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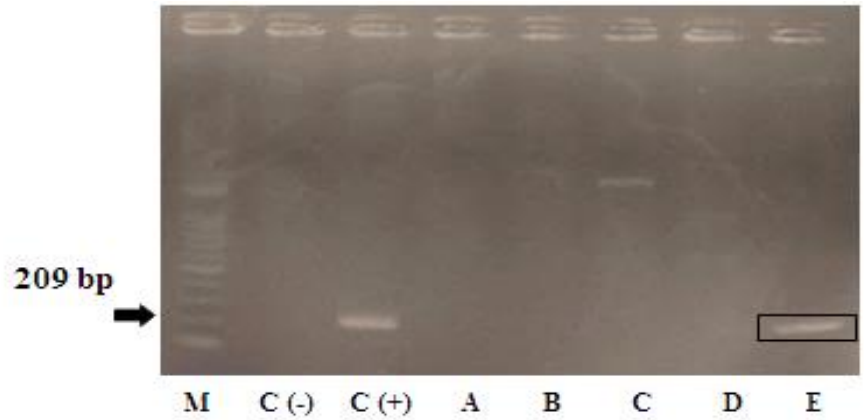


Figure 8: PCR specific test: (M) marker, (C -) negative control, (C +) positive control, (A) *S. agalactiae*, (B) *P. aeruginosa*, (C) *A. sobria*, (D) *A. salmonicida*, (E) *A. hydrophila.*

Detection of A. hydrophila antigen with co-agglutination test and PCR after injection

Detection of *A. hydrophila* with co-agglutination method compared with PCR method is shown in Table 6. After an artificial injection in gourami the presence of *A. hydrophila* was detected by co-agglutination or PCR. The fish was injected with *A. hydrophila* which invaded the fish after one hour. The co-agglutination could detect *A. hydrophila* in muscle one hour after injection. *A. hydrophila* in the internal organs (liver and kidney) was detected 12h and 24h after injection. However, PCR method was able to detect the presence of *A. hydrophila* in muscle, liver, and kidney in each test after injection.

Table 6: Detection data of *A. hydrophila* post-infection (Co-agglutination/ PCR), (+) positive reaction, (-) negative reaction, (C) co-agglutination, (P) PCR.

	Hours												Moribund fish		Dead fish	
	1		3		6		12		24		48					
Organ	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P
Muscle	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Liver	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
Kidney	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+

Discussion

Rapid detection of immunoassay methods with agglutination technique using polyclonal antibodies produced by B lymphocytes with different types of cloning due to the antigen bond response with different epitopes. The method was to inject one type of protein or antigen which was called immunogen into the body of a mammal such as a rabbit. Immunoglobulin G (IgG) was specific immunogen produced by B lymphocyte cells as an immune response. IgG would circulate in the body especially in blood serum. An adequate amount of IgG can be

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obtained from the serum of the animal (Koivunen and Krogsrud, 2006).

Agglutination ensued if the antigen component or antibody component was not dissolved. Antibody serum was made when designed particles bond with *Staphylococcus aureus* cells that had protein A. Fragment crystallizable region (Fc) of IgG bound by protein A and the antigen would stick to the specific fragment antigen-binding portion (Fab) of IgG (Wibawan and Soejoedono, 2013). The occurrence of protein A in *S. aureus* binding against rabbit antiserum is because of high affinity between Fc of IgG in rabbit and protein A (Foster *et al.,* 2014). Protein A has the ability to bind Fc of IgG in mammals but not to Fc of IgY in chicken (Xiong *et al.,* 2016).

Examination of cross reaction was important to know that *A. hydrophila* producing serum polyclonal antibody was specific to *A. hydrophila* antigen. The immunization process of rabbit was conducted by increased antibody titer value. A specific antibody need time to recognize antigen before the antibody could respond. Measurement of antibody titer in rabbits using serial dilution with antibody titer value was done from the opposite of the highest dilution that still show agglutination (Tizard, 1988).

Affecting factor of self agglutination was the chemical interactions between the components of the reagent which were created. *S. aureus* contained protein A which could bind immunoglobulin to constant regions (Fc) and variable regions (Fab) in each of the five recurrent triple-helix domains by binding to Fc domains of IgG and *Staphylococcal Immune Evasion Protein* to inhibit opsonization and phagocytosis (Bagnoli *et al.,* 2017). The purified serum binding to protein A was an important step for the success of the polyclonal antibody bonds resulting from its receptor. The impact eventually would lead to the presence of other unsuitable proteins circulating in large amounts of serum ultimately interfering with the affinity of antibody receptors, if the antibody was not purified.

The co-agglutination method in this study was done in optimized temperature and time in the antigen inactivation process before antiserum production is carried out. Pelczar *et al.* (2012) stated that temperature and time are critical to determine susceptibility of bacteria. Bacteria have proteins that are sensitive to the environment. High temperature would reduce biochemical activity of bacteria to support the antigen antibody reaction.

In this research, *A. hydrophila* antigen on gourami can be detected within 10-30sec. Other co-agglutination methods are performed by Evan (2017) to detect *Vibrio parahaemolyticus* in white shrimp within one minute, Fikar *et al.* (2015) to detect *Edwardsiella tarda* in catfish for 10min, 20min, and 30min, dan Xueqin *et al.* (1997) to detect *A. hydrophila* for three minutes.

This study has a limit detection at 10^6cfu mL^{-1} of *A. hydrophila* antigen. Ningrum *et al.* (2017) used a co-

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agglutination against *Escherichia coli* antigen to produce the lowest sensitivity level of the detectable bacteria on *E. coli* which located of 10^8cfu mL^{-1}. The specificity interaction is a major factor to increase sensitivity. The higher the percentage rate of antibody bound to protein A of *S. aureus* indicated more sensitive developed co-agglutination test. Specific antibodies occurred in the presence of specific antibody bound available on one side of Fab against one type of multivalent antigen epitope. In the other part of Fab there would be bound to another epitope in the antigen resulting in a complex antigen-antibody binding (Coico and Sunshine, 2015).

After injection of *A. hydrophila*, the cause of motile *Aeromonas* septicemia (MAS), gourami showed clinical symptoms such as, fish often on the surface of water, red spots in the mouth and around the mouth, hemorrhagic on the surface area of the body, body color not bright, and presence of ulcers and swelling at the injection site. According to Stratev and Odeyemi (2017) MAS disease would cause symptoms like, hemorrhage, ulceration and abscess on the body surface of fish, presence of fluid in the stomach, and anemia.

A. hydrophila antigen could be detected on muscle at one hour after injection. It showed that pathogenicity is started, and the bacteria continue to distribute to other internal organs such as liver and kidney. This study was supported by findings of Reddy *et al.* (2013) who measured the enzyme in liver and kidney of *Catla catla* infected by *A. hydrophila* and showed that the enzyme level present in liver was larger than that in kidney. This indicates that metabolism of enzymes found in liver was faster than in kidney due to the injection of *A. hydrophila*. In accordance with the results obtained, it could be concluded that detection of *A. hydrophila* antigen presence in liver is faster than kidney. This was caused because of presence of antigens smaller than 10^6cfu mL^{-1}. *A. hydrophila* was also found in moribund fish and dead fish. Cutuli *et al.* (2015) also showed the *A. hydrophila* was present in moribund fish and dead fish injected with *A. hydrophila*.

PCR method detected *A. hydrophila* at 209bp which was in conformity with Pollard *et al.* (1990) that used synthetic oligonucleotide primers in polymerase chain reaction (PCR) with the target of 209bp from the largest open reading framework of aerolysin gene sequence. Aerolysin gene can cause host cell apoptosis or necrosis, if there is an excess of aerolysin gene it could accelerate the process of apoptosis, ultimately causing tissue damage (Galindo *et al.* 2005).

In this study, co-agglutination method was able to detect *A. hydrophila* antigen on gourami. Sensitivity of co-agglutination had a detection limit of 10^6cfu mL^{-1}. Co-agglutination had the advantage of being able to elicit specific and senstive reaction which was rapid, accurate and requires equipment and materials that

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are relatively simple and easy to conduct in laboratory or field.

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