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## **Efficacy of agglutination test in diagnosis of motile *Aeromonas septicemia* in Nile tilapia (*Oreochromis niloticus*)**

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### **ABSTRACT**

Bacterial diseases are the most common diseases among cultured fish. *Aeromonas hydrophila* considered the most important freshwater bacterial fish pathogen. Early diagnosis considered the cornerstone in controlling the disease. Biochemical method is laborious, expensive and time consuming whereas agglutination test is simple, rapid, accurate inexpensive and the test can be performed in field conditions. In the present study, agglutination test was established for diagnosis *A. hydrophila* infection in *Oreochromis niloticus*. *A. hydrophila* was isolated from naturally infected fish then distracted to subunits somatic and flagellar antigens. The prepared antigen was reacted with positive antisera of *A. hydrophila* which produced distinct tiny clumps. The sensitivity and specificity of slide agglutination test against collected sera were 90%, 77.5% respectively in case of somatic antigen and 92.5%, 72.5% in case of flagellar antigen. It could be concluded that agglutination test can be used as screen test for diagnosis of *A. hydrophila* infection in fish farms and the method can be diagramed on other bacteria.

**Keywords:** *Aeromonas*, agglutination, antigen and *Oreochromis niloticus*

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### **Introduction**

The advent and anticipation of aquaculture is enhancing food production; more income generation and good health for the people. However, infectious diseases are the most important constraint to aquaculture development and sustainability. Production costs are

increased through investment lost in dead cultured fish, cost of treatment, and decreased quality and quantity of yields (**Idowu *et al.*, 2017**). The incidence of microbial pathogens, especially those of bacterial origin is one of the most significant factors affecting fish culture (**Zorrilla *et al.*, 2003**). *Aeromonas hydrophila* and other motile aeromonads are the most common bacteria in freshwater habitats throughout the world, (**Otte, 1963**). Bacterial infections can stem from many causes, even combinations of contributing causes. Infections can be topical (external); fin or tail rot and ulcer diseases, systemic (internal), or both. Stressed fish can be very susceptible to this illness (**Idowu *et al.*, 2017**). Latex agglutination tests have been applied in many laboratories for the detection of infectious diseases. (**Ariel and Owens 1997**) firstly described Rheumatoid Factor Test which based on latex agglutination. After that, agglutination test used to detect microbial and viral infections, autoimmune diseases, hormones, drugs and serum proteins have been developed and marketed by many companies worldwide (**Post, 1989**).

The current study was designed to evaluate the efficacy of agglutination test (slide agglutination and hemagglutination inhibition) as a serological test used in diagnosis of *A. hydrophila* infection in *Oreochromis niloticus* through preparing colored antigen from locally isolated strain.

## **Material and methods**

### **Bacterial isolate**

*A. hydrophila* strain was previously isolated from clinically diseased fish and biochemically identified in Fish Health and Management Lab. of Central Laboratory for Aquaculture Research (CLAR) according to **Austin and Austin (1987)**. Morphological and biochemical characters of the isolated *A. hydrophila* were illustrated in table (1).

### **Fish**

Total numbers of two hundreds of (50±5 g) apparently healthy Nile tilapia were obtained from production farm of CLAR. Fish were acclimated to the laboratory conditions for 2 weeks. In well equipped 10 glass aquaria (80 x 40 x 50cm) filled with dechlorinated tap water at 25±1°C and continuous aeration using air pumps in the wet laboratory fish were divided into 2 groups (G1, G2). Fish were fed at rate 3%

from the biomass and about half of the water was changed with chlorine free water and fecal matters were siphoned out once daily during the experiment. Fish were fed twice daily on standard commercially prepared pellets.

**Table (1).** Biochemical characters of *A. hydrophila* used in experimental infection.

Test	Result
Gram stain	Gram –ve Bacilli
Tryptic Soya agar	Creamy white colony
Aeromonas Base media	Blue green colony
Hemolysis (TSA with 5% sheep erythrocytes)	Beta Hemolysis
Ampicillin susceptibility	Resistant
Oxidase	+Ve
Oxidation/Fermentation	+/+
H <sub>2</sub> S	Positive
Esculin Hydrolysis	Positive
Indol	Positive
MR	Negative
VP	Positive
Mannitol fermentation	Positive
Sucrose fermentation	Positive

### Experimental design

G1, was injected with 0.5 ml intraperitoneal (I/P) saline containing bacterial isolate (*A. hydrophila*) as whole bacterial cells ( $2 \times 10^6$  cells/ml) for preparation of hyper immune serum (**Luky, 1977**). G2 left as control and injected with sterile saline. The fish were observed one week post inoculation for mortalities. The dead fish were removed daily and subjected to bacterial re-isolation to verify death. Blood was collected from all fish groups three times with 7 days interval starting from seventh day without adding anticoagulant for serum separation and evaluating the specific antibody production (**Evans *et al.*, 2004**).

### Hemagglutination inhibition test (HI)

#### Evaluation of antibody titer:

On U shape 96 well microtiter plate 50 µl PBS was added to all wells, then 50 µl of bacterial antigen was added to 1<sup>st</sup> well and double

fold serial diluted till the last one. Fifty  $\mu\text{l}$  of 0.5% washed RBCs was added to all wells, mixed by shaking plates, and incubated at room temperature for 30 minutes for measuring the bacterial cut point. The collected serum was serial diluted and tested against the bacterial cut point (McGraw, 1957).

### **Preparation subunits antigen**

#### **Somatic antigen:**

Bacterial isolate was cultured on to freshly prepared nutrient agar slope, the growth from the slope was washed with saline. Bacterial suspension was boiled in water bath for 10 minutes to destroy the less heat stable and viable antigen (Snieszko, 1976).

#### **Flagellar antigen:**

Formalin 37% was added at rate of 5 drops /10 ml broth, bacterial cells harvested by centrifugation and resuspended in normal saline (Roberts, 1989).

#### **Slide micro-agglutination test:**

##### **Antigen Staining:**

Tetrazolium blue was added aseptically, 0.5% to culture. The flask was agitated and incubated for 24 hours. The stained broth suspension was filtered through sterile gauze and poured into eppendorf tubes which were centrifuged at 14,000 rpm for 15 minutes. The supernatant fluid was decanted and the packed cells were suspended in 0.5% phenol saline. The solution was kept at 4°C as tetrazolium-stained antigen (Lowry *et al.*, 1951).

##### **Antibody evaluation:**

The collected serum was examined against stained antigen by slide micro-agglutination test. Twenty  $\mu\text{l}$  of stained antigen and 20  $\mu\text{l}$  sera were placed on clean, dry porcelain slide and mixed thoroughly. The results were read within 1 min (McGraw (1957).

##### **Estimation of sensitivity and specificity of latex agglutination test**

The analysis was performed according to (Kachigan, 1991). The test was carried out to determine the differences among treated means. Sensitivity was counted by the equation  $A/(A+C) \times 100$ , where A is true positive and C is false negative. Specificity was counted by the equation  $D/(D+B) \times 100$ , where D is true negative and B is false positive.

## Results and discussion

Bacteria are responsible for many fatal diseases in fishes. Epidemics of bacterial diseases are common in dense populations of cultured food or aquarium fish. Predisposition to such outbreaks frequently is associated with poor water quality, organic loading of the aquatic environment, handling and transport of fish, marked temperature changes, hypoxia, or other stressful conditions (**Idowu *et al.*, 2017**).

The used bacterial model in the present study was isolated from diseased fish and biochemically identified in Fish Health and Management Lab. (Table 1). The isolated bacteria was referred to *Aeromonas hydrophila* according to its biochemical characters which was the same recorded by **Austin and Austin (1987)**. So, the isolated bacteria was used in the present study for preparing of the specific serum and subunit antigens.

Experimentally infected fish showed clinical signs of the disease after 24 hrs. post inoculation of *A.hydrophila* including inflammatory changes at the site of inoculation, hemorrhages all over body surface, presence of petechial hemorrhages and congestion in liver, kidneys and spleen. Similar results were observed post re-isolation of *A.hydrophila* from all freshly dead experimentally infected fish. The mortality rate was 60% Table (2) and Table (3).

HA test is used to standardize antigens for the HI test. Hemagglutination occurs when hemagglutinins on the bacterial antigen interact with receptors on the surface of erythrocytes, and inhibition of HA occurs in the presence of specific antibodies in serum. Wells with complete hemagglutination are recorded as positive, while wells with a distinct button formation are recorded as negative. The endpoint of the titration is the highest dilution of antigen causing complete hemagglutination. The hemagglutinin endpoint was 1/32 and hemagglutinins unit was 32 (Photo 1).

HI test is used to detect and quantitative specific antibodies in serum. Endpoints are reported as the highest serum dilution causing complete inhibition of hemagglutination. The titer was gradually increased following the artificial infection and the highest antibody titer was observed in day 21 post inoculation (Table 4 and Photo 2). Similar findings were described by **Sahu *et al.*, (2007)**.

The use of subunit flagellar and somatic parts in the antigen is due to protein of this portions are similar with wide range of the *A. hydrophila* serotypes and it is easy to identified at this level that was cited by **Evans *et al.*, (2004)** who recorded many protein molecules are composed of more than one subunit, where each subunit is a separate polypeptide chain and can form a stable folded structure by itself. The amino acid sequences can either be identical for each subunit, or similar (as in  $\alpha$  and  $\beta$  chains of hemoglobin). The assembly of many identical subunits provides a very efficient way of constructing large structures such as bacterial coats. Often a multi-subunit molecule is more smoothly globular than its component subunits.

Colored stained antigen was prepared (Photo 3). The total protein concentrations of stained subunit flagellar and somatic antigens were adjusted to 6.4 ng/ $\mu$ l with the help of standard curve at this level can aid in efficient.

There is a need for specific diagnosis of bacterial diseases due to a number of bacteria produce a similar syndrome, generically referred to as hemorrhagic septicemia and characterized by external reddening and hemorrhage in the peritoneum, body wall, and viscera. Morbidity and mortality are highly variable, depending on predisposing conditions such as low dissolved oxygen, other water quality problems, handling stress, or trauma. Ulcerative lesions are common as disease progresses, and mortality can be significant if stress is not controlled **Zorrilla *et al.*, (2003)**.

The serum collected after the artificial inoculation of experimented fish was examined against stained antigen by slide micro-agglutination test. The serum was having different immune titer due to it was collected at wide range time and it was giving good indication about the ability of this sainted antigen to be diagnosed the *A. hydrophila* infection and the ability was related to the produced immune level (Table 5). The antibody titer was suitable for agglutination process and can be detected the infection even at low level and same results was recorded by **Roberts, (1989)** who cited there is no agglutination can be observed when the concentration of antibody is high, (lower dilutions), and then the sample is diluted, agglutination occurs. Prozone effect is defined as the invisibility of agglutination at high concentrations of antibodies. It is due to the

reason that excess antibody forms very minute complexes that do not clump to form visible agglutination.

Methods of detecting quantitative antigen or antibody take advantage of the fact that they react to form a complex. At the optimum antigen-antibody concentration, this complex precipitate out. However, if the antigen is particulate in nature, agglutination of antigen-antibody complex is observed (**Roberts, 1989**).

The reaction between a particulate antigen and an antibody results in visible clumping called agglutination. Antibodies that produce such reactions are known as agglutinins. The principle of Agglutination reaction is similar to precipitation reactions; they depend on the cross linking of polyvalent antigens. When the antigen is an erythrocyte it is called hemagglutination. Theoretically all antibodies can agglutinate particulate antigens (**Pridgeon and Klesius 2011**).

The examined serum samples from artificial infected fish with *A. hydrophila*, experimentally induced, reacted strongly in the latex test (Photo, 4). Moreover, serum from healthy fish showed negative. In addition to permitting rapid and accurate diagnosis of *A. hydrophila*. The sensitivity and specificity of the method were found to be 90%, 77.5% (Table 6) in case of somatic antigen and 92.5% 72.5% in case of flagellar antigen respectively (Table 7). The Sensitivity and specificity gave an idea about the good efficiency of the stained antigen to diagnosis the disease and that similar with that recorded by (**Kachigan, 1991**) who cited sensitivity and specificity describe how well the test discriminates between variables with and without disease. They address a different question than want answered when evaluating variables.

Common bacterial isolates from affected fish include *Aeromonas* and *Pseudomonas* sp., which are more common in freshwater animals, and *Vibrio* sp., more commonly isolated from marine fishes. Control is based on quick removal of predisposing factors by rapid field diagnosis.

It can be concluded that using of established latex can be easy applied as agglutination test in field diagnosis of some bacterial fish.

**Table (2).** Mortality rate of experimentally infected fish with isolated strain of *A. hydrophila*

<b>Group</b>	<b>Total No.</b>	<b>No of dead</b>	<b>Mortality rate</b>
<b>G1</b>	100	60	60%
<b>G2</b>	100	5	5%

G1 injected with *isolated strain of A. hydrophila*

G2 (Control) injected with sterile saline

**Table (3).** Number of dead fish per day during the first week post inoculation

<b>Days post inoculation</b>	<b>1<sup>st</sup></b>	<b>2<sup>nd</sup></b>	<b>3<sup>rd</sup></b>	<b>4<sup>th</sup></b>	<b>5<sup>th</sup></b>	<b>6<sup>th</sup></b>	<b>7<sup>th</sup></b>
<b>G1</b>	7	30	15	5	3	-	-
<b>G2</b>	3	2	-	-	-	-	-

G1 injected with *isolated strain of A. hydrophila*

G2 (Control) injected with sterile saline

**Table (4).** Antibodies titers measured by hemagglutination inhibition test

<b>Time</b>	<b>7 days</b>	<b>14 days</b>	<b>21 days</b>
<b>Antibody titer</b>	8	32	128

**Table (5).** Examination of stained antigens against different levels of prepared specific antibodies

<b>Antibody titer</b>	<b>8</b>	<b>32</b>	<b>128</b>
<b>Agglutination by stained Ag</b>	91%	94%	96%

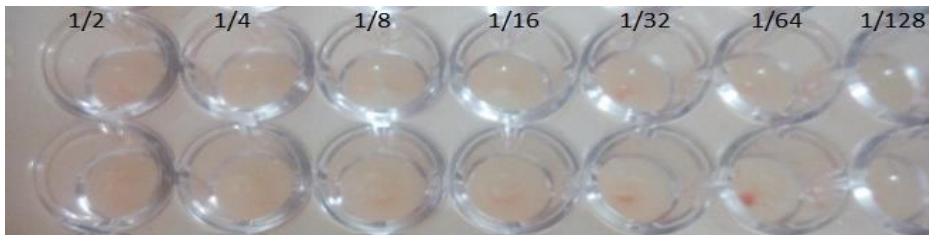
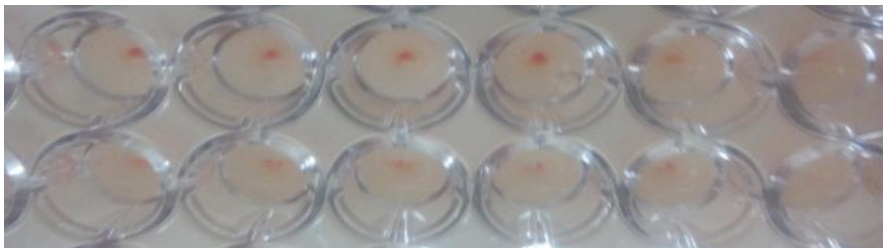


**Table (6).** Sensitivity and specificity of slide agglutination for somatic antigen of *A.hydrophila*

	<b>G1</b>	<b>G2</b>	<b>Sensitivity</b>	<b>Specificity</b>
<b>Positive</b>	36	9		
<b>Negative</b>	4	31	90%	77.5%
<b>Total No.</b>	40	40		

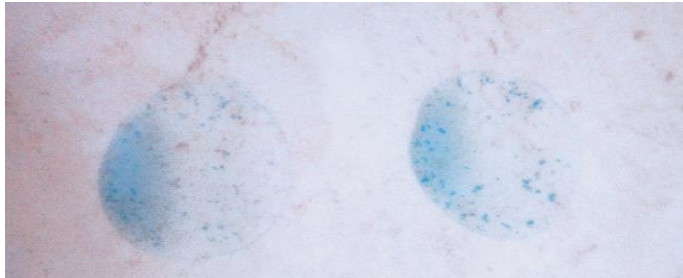
**Table (7).** Sensitivity and specificity of slide agglutination test for flagellar antigen of *A.hydrophila*

	<b>G1</b>	<b>G2</b>	<b>Sensitivity</b>	<b>Specificity</b>
<b>Positive</b>	37	11		
<b>Negative</b>	3	29	92.5%	72.5%
<b>Total No.</b>	40	40		

**Photo. (1).** Hemagglutination activity of isolated *A. hydrophila*.**Photo. (2).** Hemagglutination inhibition activity of serum collected from artificial infected tilapia.



**Photo. (3).** Stained colored antigen of locally isolated *A. hydrophila*.



**Photo. (4).** Slide agglutination test showing strong react of *A. hydrophila* colored stained antigen with serum samples of experimentally infected fish.

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## كفاءة اختبار التخثر في تشخيص مرض التسمم الدموي الإيروموناتى فى أسماك البلطى النيلى

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### الملخص العربى

تعتبر بكتيريا الإيرومونات هيدروفيليا من أهم المسببات المرضية في مزارع الأسماك خاصة أسماك المياه العذبة ومن هنا كان من الضروري العمل على السيطرة على هذه البكتيريا حيث يعتبر التشخيص المبكر لها أساس العمل على الحد من الخسائر التي قد تسببها في الوقت الذي يعتبر فيه التشخيص من خلال العزل والتصنيف باهظ التكلفة ومستهلك كثيرا من الوقت والجهد على الأقل بالنسبة لمربي الأسماك.

ومن هنا كان الهدف من هذه الدراسة هو عمل كاشفات بسيطة من السهل أن تستخدم في أماكن إنتاج الأسماك بالإضافة إلى سعرها الرخيص.

ولهذا كان الهدف من هذه الدراسة عمل نموذج تشخيص بسيط يعتمد على التلازن بين بكتيريا الإيرومونات هيدروفيليا والأجسام المناعية في عينات دم مجمعة من الأسماك والتي أظهرت كفاءة عالية ونجاح عند استعمال تلك الكاشفات في تشخيص هذه البكتيريا في الحقل إذ كان درجة كفاءتها أكثر من 90% ومن هنا توصى الدراسة باستعمالها للكشف السريع ومن ثم السيطرة السريعة على هذه البكتيريا كما أنه من الممكن تطبيق هذه الطريقة في عمل كاشفات مشابهة للكشف على أنواع أخرى من البكتيريا.