Abbassa Int. J. Aqua. Spiceal Issue 2010
The third scientific Conference, Al Azhar University, Cairo 17 –18 October 2010

EVALUATION OF SOME IMMUNOSOROLOGICAL TESTS FOR IDENTIFICATION OF *AEROMONAS SOBRIA IN COMMON CARP (CYPRINUS CARPIO)*

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Abstract

Specific immune serum was prepared from common carp (*Cyprinus carpio*) which has been immunized with formalized bacterin of *Aeromonas sobria* (*A. sobria*). This serum used as guide reagent for investigation of *A. sobria* infection through sero – immunlogical tests as immune phagocytosis, bactericidal, slide agglutination, agglutinin titration and this was the aim of this study. Results of immune phagocytosis gave general indication about infection. Bactericidal test was reveled growth inhibition of *A. sobria* on tryptic soya agar plate after 48 hr of incubation. Strong agglutination of bacteria within 2 min was the result of slide agglutination and the highest titer of agglutinin titration was 1/160. The indirect fluoresce antibody technique revealed a shine florescence isothiocyanate light. Histopathological examination was done as confirmatory diagnostic methods. So, it is suggested that immune serum against some bacteria may be used as early accurate diagnostic tools for successful treatment of bacterial diseases.
Key wards: common carp, *A. sobria,* immune serum, agglutinin titration, immune phagocytosis, bactericidal, slide agglutination

Introduction

Infectious bacterial fish diseases and infections are very common in fish and probably one of the hardest health problems to deal with that problems effectively. However, with a basic understanding of how bacteria cause disease and how to recognize bacterial infections it is possible to deal successfully with the majority of problems. The key for successful treatment of bacterial disease is early and accurate diagnosis (Kimmi *et al.,* 2008).

To speed up identification and increase accuracy, serological and biochemical procedures are being used to detect some pathogen in situ and in vitro procedures use direct and indirect fluoresce antibody, several varieties of ELISA, radio labeling and polymerase chain reaction (Schill *et al.,* 1989). The rapid, highly sensitive procedures can be benefited in diagnosis of infectious fish diseases specially those associated with bacteria. Several related *Aeromonas spp*, *Aeromonas hydrophila, Aeromonas caviae, Aeromonas sobria* (*A. sobria*) and

Aeromonas veronii were identified as the causative agent of fin and tail rot allover the world. The organisms, identified as *A. sobria,* have been isolated during 1987 (Toranzo *et al.,* 1989) and consider the causative agent of skin lesions and fin rot in Switzerland farmed fish (Wahli *et al.,* 2005). It is possible that *A. sobria* may be the primary cause of MAS infection in cultured channel catfish (Plumb1994). It may be capable of causing illness in humans by infecting open wounds and possibly by ingesting the organisms in food or water (Datta *et al.,* 2008).

Definite diagnosis of bacterial fish disease requires sampling and culture of bacteria from the lesion or organ. The problem is that many clinical signs of bacterial disease could also be caused by other pathogens such as viruses, poor water quality or parasites and so, need to be aware of the dangers of jumping to conclusions (Bruhn *et al.,* 2005).

The key for successful treatment of bacterial disease is early, accurate diagnosis. If treatment is delayed it can lead to substantial losses so, the present study was undertaken to estimate an accurate specific and quick method for diagnosis of *A. sobria* infection using specific immune serum.

Material and Methods

Virulence of *A. sobria* for Common Carp

One hundred and six common carp (weighted 30-40 gr.) were acclimated in 11 glass aquaria (200 L.) capacity provided with dechlorinated tap water daily renewal for one week. They fed a 25% crud protein ration at 1,5% biomass two time daily

A. sobria kindly provided by Fish Diseases Dept. of (CLAR) was cultured on nutrient broth at 37 C for 24 hr, the bacterial cells was suspended and adjusted to be $6x10^8$ CFU/ml and diluted in two fold serial dialution. Sixty fishes divided into 6 equal groups, injected I/P (2 replicate)and I/M(2 replicate) with 0.5ml of bacterial suspension and 2 groups injected with 0.5 ml saline as shown in table (1).

Antigen Preparation

Whole *A. sobria* cells ($6x10^8$ CFU/ml) was inactivated by using 0.3% formalin (v/v). The technique was done according to (Caron & Meyer, 1989).

Table (1): Evaluation of *A. sobria* Virulence

Group	No. of Fish	Inoculated Rout	M.O	Dose
1	10	I/P	*A. sobria*	0.5 ml $6x10^8$ CFU/ml
2	10	I/P	*A. sobria*	
3	10	I/M	*A. sobria*	
4	10	I/M	*A. sobria*	
5	10	I/P	Saline	0.5 ml
6	10	I/M	Saline	

Immune Serum Preparation

Twenty common carp injected I/P with 0.5 ml 6x10 CFU/ml Formalized bacterin of A. Sobria was applied by i.p. injection three times one week interval. The blood was collected from caudal vein of previously anesthetic fish by clove oil at a concentration (74.3 mg/L) according to (velisek *et al.,* 2005) after 21 days from the first inoculation without anticoagulant and centrifuged for 20 min. at 3000 rpm to obtain the serum for immune serum preparation. According to (Silverstein, 1989).

Negative Serum

Blood was collected from non inoculated other 20 fish and used as negative guide in the techniques as described befor.

Immune Phagocytosis

0.5 ml of prepared immune serum (diluted 1/10 in sodium chloride 0.85 %) mixed with bacterial cells of *A.sobria* at a concentration 6x10 CFU/ml (suspended bacterial slant in sodium chloride 0.85 % by 1:2 ml) and injected I/P into apparently health 3 fishes of common carp maintained in glass aquarium as mentioned before. Other 3 fishes were injected by same dose but a mixture of bacteria cells with negative serum and the fishes kept as control. After 1 hr the injected fishes were sacrificed, opened the peritoneum and extracted the contented fluid and by using loop smear contented onto slides. The slides were stained with Gemisa stain and observed microscopically. The technique was done according to (Gudding *et al.,* 1999).

Bactericidal Test

0.5 ml of immune serum was serially two fold diluted by PBS mixed with 0.5 ml of *A. sobria* strain at concentration of $6x10^8$ CFU/ml and incubated for 1 hr. at 37 °C then cultured on tryptic soya agar for 48 hr. and observed for the growth of

the bacteria on the agar. Control negative serum was used by the same way according to (Benmansour & de Kinkelin, 1997).

Slide Agglutination

1 ml of phenol saline was added to each of agar slant culture and suspended the growth of bacteria. With capillary pipette one drop of each bacterial suspension was added to opposite ends of the slides. One drop of prepared antiserum diluted 1:10 was added to each of the bacterial suspension and left in the room temperature. The slides were examined microscopically for detection of agglutination. The control was done by mixing drop of bacterial suspension and drop of negative serum. The technique was done according to (Mokhlasur *et al.,* 2002).

Agglutinin Titration

Immune serum was serial two fold diluted and titerated against suspension of *A. sobria* strain $6x10^8$ CFU/ml then mixed well. The mixture was incubated over night at 37◦C and recorded the results. The technique was done according to (McGraw, 1957). The control was done by using two fold dilution of negative serum.

Indirect Fluoresce Antibody Technique (iFAT)

Slides smeared with isolated bacteria fixed with acetone 80 % for 10 minutes. 1st specific and 2nd labeled antibodies were added. Incubation was done at 37◦C for 30 minutes in humidified atmosphere then washed three times in petridishes containing PBS and dried on filter paper. The slides were examined under fluorescence microscope after mounted in a pre-warmed mounting glycerin buffer according to (Marsden *et al.,* 1996).

Histopathological Examination

For histopathological examination of artificially infected tissues, specimens were collected from liver, gills and kidney. The specimens were fixed in 10% neutral formalin, embedded in paraffin, sectioned and stained with Hematoxyline and Eosin according to method described by (Drury *et al.,* 1976).

RESULTS

Mortality Rates

Common carp mortality rates inoculated with *r. Sobria* showed increase the mortality percent by I/p rout injection than I/M one as shown in table (2).

Table (2): Mortality Rates among Carp Fish Inoculated with *A. Sobria*

Gr.	No.	IMO	RI	No. of Fish during 15 Days															DF	SF	%
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			
1	10	*A.sobri*	I/P	-	-	-	-	2	3	-	1	1	-	-	-	1	-	-	8	2	80
2	10	*A.sobri*	I/P	-	-	-	-	4	3	-	1	-	-	-	1	-	-	-	9	1	90
3	10	*A.sobri*	I/M	-	-	-	-	1	1	-	-	1	-	1	-	-	1	-	5	5	50
4	10	A.sobria	I/M	-	-	-	-	-	-	-	1	-	-	1	-	-	1	-	3	7	30
5	10	Sterile broth	I/P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	10	Sterile broth	I/M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Gr: group No. Number MO: Inoculated Micro-organism

RI: Rout of inoculation DF: Dead Fish SF: Survival Fish

Clinical Finding and Post Mortem Examination

The artificially infected carp under investigation showed; loss of balance, loss of appetite, sluggish movement, tail rot, enlarged pale liver beside congested kidney and gills.

Immune Phagocytosis

Three films of each injected fish (both experimental and control) were smeared by The extracted peritoneal fluid stained with Gemisa and observed microscopically with the oil emersion lens and recorded the number of monocytes in 10 HPF.The results showed relative increase of monocytes infiltration in peritoneal fluids of negative serum compared with that of immune serum.

Bactericidal Test

The prepared immune serum double folds titerated and mixed with *A. sobria* strain then cultured on tryptic soya agar. Bacterial growth was observed during 48hr. Examination of plates showed inhibition for the bacterial growth on the agar at high serum titer and growth appeared at low titer as showed in table (3). While, control negative serum with bacteria showed growth at all titer.

Table (3): Showing Bacterial Growth against Immune Serum Titration

Serum titer	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
Bacterial growth	NG	NG	NG	NG	NG	RG	RG	G	G

NG: no growth G: growth RG: relative growth

Slide Agglutination

Examination of the slides under the low power of the light microscope showed clumping of the bacteria in the positive cases. The time elapsed for agglutination was observed within two minutes (Fig.1&2).

Agglutinin Titration

When the incubation was completed, the tubes were removed from incubator and recorded the agglutination as demonstrated in table (4) and (Fig. 3)

Table (4): Showing Results of Agglutinin Titration

Tube No.	1	2	3	4	5	6	7	8	9
Immune Serum dilution	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
Agglutinin	+++	+++	+++	+++	+	+/-	-	-	-
Negative Serum Dilution	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
Agglutinin	-	-	-	-	-	-	-	-	-

Indirect Fluoresce Antibody Technique

Slides smeared with isolated bacteria fixed with acetone, 1st and 2nd labeled antibodies were added. Incubation was done then washed three times. The slides were examined under fluorescence microscope. Our results showed appearance of shine fluorescence isothiocynate light due to reflection of UV light on the attached conjugate. (Fig. 4)

Histopathological Examination

The gills showed mild to moderate hyperplasia while, some cases showed desquamates of the epithelial cells, dilatation of bronchial blood vessels, hemorrhage, edema and leukocytic infiltration in gill arch. Fig (5&6). Liver showed sligint congestion of hepatic blood vessels with few lymphocytic infiltrations. Kidney showed condensed glomeruli (Fig. 7), focal congestion and hemorrhages, some cases showed detachment of epithelial cells lining the renal tubules.

Discussion

Defined accurate diagnosis of fish disease is way for treatment of the infection and as soon as possible rapid diagnosis was done, meaning rapid treatment will be done. The developments of serological methods for the detection and/or diagnosis of bacterial infection must be examined to avoid wide range of false positive reactions and false negative reactions (Pascho *et al.,* 1998). So that, we used many serological testes for investigation a rapid diagnosis for bacterial infection.

Immune phagocytosis technique was done which showed infiltration of immune cells mainly monocytes in abdominal cavity giving an indication about presence of invasive agent (Dueholm *et al.,* 1998). It can be used as permeably test for diagnosis and not consider as main diagnostic technique, because it gave a general idea about presence of infection in acute stage and not determined the specific type of the infusive. In our opinion, the results reveled relative increase of monocytes in the control group than test group and this may be returned to the immune serum inhibited the antigensty of the injected bacteria while, the antigensty was not affected and so, more monocytes migrated from blood to tissue to control the virulent microorganism.

Bactericidal test was done as specific test for identification type of bacterial in which specific serum and the isolated bacteria were incubated with each other on the bacterial media. The specific serum was inhibiting growth of investing bacteria (*A. sobria*) and that was accepted by (McGraw, 1957). The technique used as easy applied specific test and can determined the type of invasive organism.

Slide agglutination and agglutinin titration are quick, easy technique, provide much useful data can be used widely as diagnostic tool for fish pathogens (Kitao, 1982) and so, we used the two technique for the same aims, the slide agglutination showed clumping of the bacteria on the used slide in the time ranged between 2 – 8 min according to bacteria and serum concentration. Similar results were recorded by (Kitao, 1982).

(Rabb *et al.,* 1964) considered the technique is effective with most of bacterial colonies. The agglutinin titration test was used as tool for determination of the specific immune response level against the infection in which twofold serial

dilution was done for the serum incubated with the bacteria and determined the cut off point of agglutinin which was at 1x 160 and this reported by (McCarthy, 1976). The application of agglutinin titration test has many benefits; by it can know healthy state of the fish and the majority of recovered from clinical cases of disease. In comparing between slide agglutination and agglutinin titration (Eurell *et al.,* 1978) showed the effectiveness of slide agglutination especially for field use in the recognition for many bacterial infections, whereas tube and macro-agglutination were useful in laboratories.

Also, indirect fluoresce antibody technique was done in which positive results were appearance of shine fluorescence isothiocynate light at reflection of UV light on the attached conjugate. Similar observed by (Silverstein, 1989). iFAT appears to be useful for detecting of fish bacterial infection and has a proven track record with the diagnosis and recognition of the bacteria in fish tissues. (Pascho *et al.,* 1998) compared between serological techniques and found FAT was capable of detecting >25 bacterial cells/ml of ovarian fluid, whereas the ELISA was not consistent in detection at levels of < 1.3 x 10^ cells/ml and recorded FAT has found use for the diagnosis of many fish diseases, especially in laboratories.

Histopathological examination alteration in gill, liver and kidney gave us an idea about the virulence of used bacteria. Similar Histopathological changes were observed by Robert, 2001

Conclusion: The present study refer to that seroimmunlogical tests may be used as a rapid accurate diagnosis tools for some bacteria diseases as subsequence rapid treatment.

Figures

Fig. (1) Showing clumping of *Aeromonas sobria* antigen with the specific antibodies

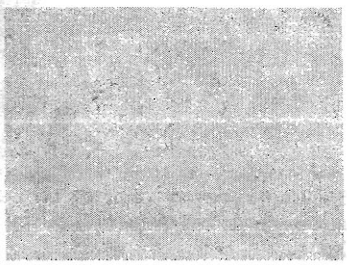

Fig. (2) Showing negative control of slide agglutination showing no clumping

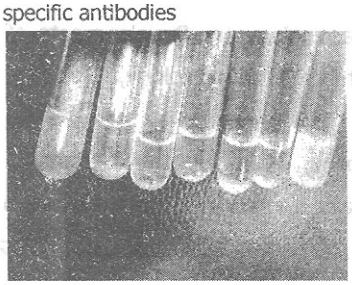

Fig. (3) Agglutinin titration showed the degree of agglutination with serial serum dilution

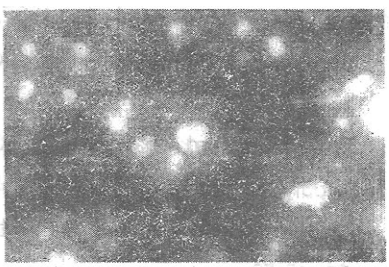

Fig. (4) Showing shine fluorescence isothiocynate light due to reflection of UV light on the attached conjugate. **Xp 400**

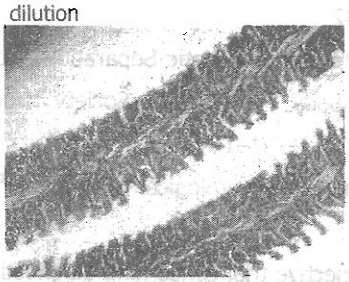

Fig. (5) Showing gills of common carp artificially inoculated with 0.5µl $6x10^8$ CFU/ml showed hyperplasia. **Xp 400**

Fig. (6) Showing gills of common carp artificially inoculated with 0.5µl $6x10^8$ CFU/ml showed congestion and hemorrhage. **Xp 400**

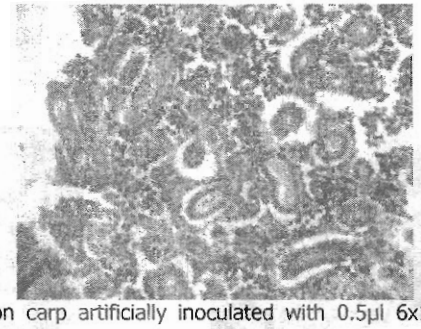

Fig. (7) Showing kidney of common carp artificially inoculated with 0.5µl $6x10^8$ CFU/ml showed congested glomeruli .
Xp 400

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تقييم بعض الإختبارات المناعية لتشخيص الإصابة بميكروب الإريموناس سوبريا فى أسماك المبروك العادى

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قسم أمراض الأسماك ورعايتها

الملخص العربى

تهدف هذه الدراسة لعمل مقارنة بين الطرق التشخيصية المختليفة لمعرفة أفضل وأدق وأسرع طريقة لتشخيص الأصابة بميكروب الإريموناس سوبريا فى أسماك المبروك العادى. ولهذا الغرض تم تحضير أجسام مناعية متخصصة مضادة لبكتيريا الإريموناس سوبريا فى دم المبروك العادي وذلك من خلال حقنها بتلك البكتيريا بعد قتلها بالفورمالين وكان الغرض من هذا هو استخلاص المصل من أسماك المبروك واستعماله فى التشخيص المناعى لتلك البكتيريا فى اختبارات: الكشف عن وجود الخلايا البالعة، الإختبار القاتل لنمو البكتيريا، اختبار التلازن على الشريحة، قياس المستوى المناعى فى دم الأسماك المصابة.

وقد أعطى اختبار وجود الخلايا البالعة صورة عامة عن الوجود المرضى فى الأسماك من خلال التواجد الكثيف لخلايا الدم المناعية، وتوقف نمو البكتيريا أثناء زرعها مع المصل الضاد لها على وسط التربتك صويا حتى ٤٨ ساعة من التحضين، وتم تسجيل تلازن شديد من البكتيريا بعد دقيقتين على شريحة التحضير، وفى اختبار القياس المناعى تم تسجيل مستوى مناعى١/١٦٠ كما تم إجراء إختبار المناعى الفلورسنتى غير المباشر وفحص الأنسجة المصابة للأسماك وذلك كاختبارات تأكيديه أثناء الاستكشافات المرضية.

ومن هنا كان اقتراح استعمال أمصال متخصصة للتشخيص السريع لأمراض الأسماك وذلك حتى يصحبه علاج سريع للمرض.