TRIAL FOR PREPARATION OF CHLOROFORM KILLED CELL BACTERIN AGAINST INFECTION WITH PHOTOBACTERIUM DAMSELA SUBSPECIES PISCICIDA ISOLATED FROM SEA BREAM (SPARUS AURATA)

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ABSTRACT

Photobacterium damsela subsp. *piscicida* was isolated from 18.00 % diseased sea bream (Sparus aurata). Rate of isolation was high from kidneys (18.00 %) followed by liver (12.00 %), spleen (10.00 %) and intestinal contents (6.00 %). Locally chloroform killed cell bacterin was prepared and proved to be effective in the immunization of Mugil cephalus against the disease where it produced agglutinating antibodies titres 37.3±0.7 post-primary vaccination and 459±0.341 post-secondary vaccination with protection rate of 75.00 %.

INTRODUCTION

Pasteurellosis or pseudotuberculosis caused by *Pasteurella piscicida* is one of the common bacterial diseases causing serious economical losses in many species of wild and farmed marine fish worldwide in Asia, USA, Europe, Mediterranean countries, and it is a very important disease in yellowtail seriola quinqueradiata culture in Japan (**Kitao, 1993, and Romalde and Magarinos, 1997**).

Infectivity trials showed that P.piscicida did not possess strict host specificity since the majority of the isolates were virulent for gilthead seabream, rainbow trout and turbot, with LD 50 values ranging between 10^3 and 10^6 live cells (**Magarinos *et al.*, 1992**).

The causative agent of fish pasteurellosis is an organism formerly known as *Pasteurella piscicida.* It has been reclassified as *Photobacterium damsela* subsp. piscicida on the basis of 16 S rRNA gene sequence comparisons and chromosomal DNA- DNA hybridization data (**Osorio *et al.*, 1999**).

The organism appears as Gram negative rods showing bipolar staining and pleomorphism. No spores are observed. Non-motile and produce viscid colonies. Growth is observed in a temperature range of 20 to 30°C and the

salinity range of growth is between 0.5 % and 4.0 %. It is aerobic and facultative anaerobic. Oxidase and catalase are produced. Nitrates are not reduced to nitrites. Lysine and ornithine decarboxylases are not produced but arginine dihydrolase is produced. The egg yolk reaction is positive. Tween 80 and tributyrin are decomposed. Phosphatase is produced. Beta haemolysis is revealed onto a medium containing defibrinated blood from chickens or carp but not from mammals. Methyl red and Voges-Proskauer tests are positive, and acetoin is produced from 2,3-butanediol. Glucose is fermented without gas production. Acid is produced from fructose, mannose, galactose, sucrose, maltose, dextrin and glycerol (**Koike *et al.*, 1975**).

The aim of this study was made to spot light on the isolation and complete identification of *Photobacterium damsela* subsp. *piscicida* from diseased sea bream. Evaluation of the immune response of Mugil cephalus to the locally prepared vaccine by detecting the antibody levels and conducting bioassay.

MATERIAL AND METHODS

A total of 50 moribund sea bream showing dark skin pigmentation, ascites in the abdomen, erosion of tail, and loss of scales were collected from floating cages of the Egyptian Company for fishing and its equipment at Matrouh Governorate. P.M. examination of these fish showed paleness of liver with enlargement of gall bladder and typical white tubercles of 1 – 2 mm in diameter in kidneys as shown in Fig. (1).

Liver, spleen, kidneys and intestine of the collected fish were examined bacteriologically. Cultivation was done in tryptone soya broth supplemented with sodium chloride at a final concentration of 2.00 %. The inoculated tubes were incubated at 20 – 22°C for 48 – 72 hours. Subculture was made onto tryptone soya (TS) agar containing 2.00 % NaCl. The inoculated plates were incubated as mentioned previously.

The resultant bacterial isolates were purified and identified according to **Koike *et al.*, (1975)**. Bacterial films were prepared and stained with Gram's stain to show the characteristic morphology of the organism as shown in Fig. (2).

Preparation of the vaccine:

Chloroform killed bacterin was prepared according to **Kawakami *et al.*, (1997)**. Bacteria were cultured in 2.00 % NaCl-BHI (Sodium chloride-Brain Heart Infusion) broth at 25°C for 48 hours, and the culture medium was centrifuged at 5000 g for 30 minutes. The supernatant was removed and inactivated by the addition of 0.1 % formalin. Bacterial cells were killed by means of 0.5 % chloroform-PS solution at 4°C for 24 hours. Chloroform layers were removed by evaporation at 50°C for 30 minutes. Killed bacterial cells were collected by centrifugation at 5000 g for 30 minutes, then added to

the inactivated culture supernatants. Bacterial concentration was adjusted to 2 x 10^9 cells/ml (McFarland opacity tube standard No. 7).

The produced vaccine was tested for purity and sterility by culturing onto the surface of tryptone soya agar plates containing 2.00 % sodium chloride. The inoculated plates were incubated aerobically at 22°C for 72 hours. Biological safety of the vaccine was studied according to **Sandhu, (1991)**.

Vaccination:

A total of 30 Mugil cephalus were used in this study. 20 of them were vaccinated and the remaining were kept as a control. Each one of the vaccinated fish was injected intraperitoneally with 0.5 ml of the bacterin. Control fish were injected with the same volume of sterile phosphate buffered saline. Vaccinated and control fish were maintained in fiberglass containers supplied with sea water at 23.0 ± 2.5°C. Three weeks later, fish were re-vaccinated to investigate the effect of booster vaccination on fish protection against pasteurellosis.

Agglutinating antibodies in fish sera:

Two weeks post-primary vaccination and 2-weeks post-booster dose, sera were collected from half of the vaccinated and control fish (10 fish from the vaccinated group and 5 fish from the control ones). Agglutinating antibodies titre against formalin killed antigens were conducted using standard micro-agglutination procedure as described by **Roberson, (1990)**. The initial dilution was made 1/10, then serial 2-fold dilutions were made (1/20, 1/40, 1/80, 1/160.... and so on). Geometric mean ± standard error of the studied sera was determined according to **Purchase *et al.*, (1989)**.

Experimental challenge procedure:

Two weeks post-secondary vaccination, the other half of fish in each group were challenged by immersion into seawater containing live culture of Ph. damsela ss. piscicida at 22°C for 5 minutes. The challenge doses used were $10^2 - 10^4$ CFU/ ml. Fish were observed for up to 10 days. Fish that died were necropsies and their internal organs (liver, spleen and kidneys) were collected and inoculated onto 2.00 % NaCl-TS agar plates to determine the presence of Ph. damsela ss. piscicida (**Kawakami *et al.*, 1997**). Potency of vaccine was calculated as relative percent survival (RPS) using the following formula:

RPS = (1 - % vaccinated mortality / % non- vaccinated mortality) x 100 according to Amend (1981).

RESULTS AND DISCUSSION

As shown in Tables (1 & 2), *Photobacterium damsela* subsp. piscicida was found to be the cause of infection in 18.00 % of the diseased seabream (Sparus aurata). The isolation rate was high from kidneys (18.00 %) followed by liver (12.00 %), spleen (10.00 %) and intestinal contents (6.00 %). **Candan *et al.*, (1996)** recorded the first outbreak of pasteurellosis in seabass cultured during September and October 1994 on a land based marine fish Farm at the North Aegean Coast of Turkey. A few affected fish developed dark body colour and some had enlarged spleen with whitish tubercles. Total cumulative mortality in all ponds during 2-weeks period was about 9.00 % (18/250 fish). **Bakopoulos *et al.*, (1997)** recorded a pasteurellosis outbreak in an offshore cage farm for gilthead seabream (Sparus auratus, Pagrus aurata) in Malta. The pathogen entered the farm with juvenile fish and the disease occurred within 2 months of their arrival. Losses first affected small fish (10 g) and then affected large fish (280 and 325 g). Mortalities were low (1.5 % in small fish and 0.2 % in large fish). **Zorrilla *et al.*, (1999)** reported the first outbreak of pasteurellosis affecting sole, Solea senegalensis (Kaup) cultured in the South-West of Spain. On the other hand, **Magarinos *et al.*, (1996)** found that the isolates of Ph. damsela subsp. piscicida had a great binding capacity to intestines from the marine fish hosts gilthead sea- bream, seabass and turbot with values ranging from 10^4 to 10^5 viable bacteria/g.

As shown in.Tables (3, 4 and 5), the level of agglutinating antibodies titre of vaccinated Mugil cephalus with chloroform killed bacterin was moderate post-first dose of vaccine (37.3 ±0.7) and markedly increased after the booster dose (459±0.341). In contrast, agglutinating antibodies in control fish were very low (13.8±0.244). Moreover, experimental challenge of Mugil cephalus fish with a live culture of *Ph. damsela* subsp. *piscicida* showed 10.00 % mortality in vaccinated fish and 40.00 % mortality in control fish with 75.00 % vaccine potency as indicated by relative percent survival (RPS). As a confirmatory, the organism was isolated from the internal organs of vaccinated as well as control dead fish. **Magarinos *et al.*, (1994)** recorded that the toxoid-enriched whole cell vaccine (WCEB) conferred good protection in gilthead seabream against Ph. damsela subsp. piscicida by bath immersion within a 4-week period. The relative percent survival (RPS) achieved with this type of vaccine ranged between 37.00 % and 41.00 % depending on the bacterial strain and dose used in the challenge. The booster immunization did not increase the protection levels of gilthead seabream. Failure of aeration system before the booster vaccination experiments could be, in part, responsible for this result. The antibody response in the sera of immunized fish was very low with no correlation between the level of agglutinating antibodies and the protection. With the high bacterial challenge doses, the mortality of the vaccinated fish was 42.50 – 45.00 % compared with the mortality of the non-vaccinated fish (72.50 – 75.00 %). The antibody

response was very low (titre of 1:8). **Kawakami *et al.*, (1997)** recorded that vaccination of yellowtail (Seriola quinqueradiata) with lipopolysaccharide mixed chloroform killed cell (LPS-CKC) bacterin stimulated the production of serum agglutinating antibodies against the organism. The serum agglutinating titre in the vaccinated fish was 16 – 128. No agglutinins were detected in the sera of control fish. Fish vaccinated with the LPS-CKC bacterin showed a relative percent of survival rate reaching 60.00 % or greater when challenged by the immersion method and the efficacy of this bacterin continued for at least 35 days post-vaccination.

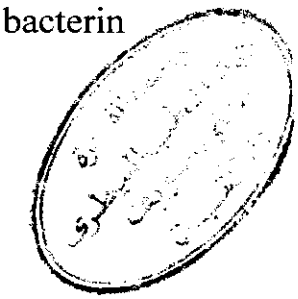

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Table (1): Recovery rate of *Photobacterium damsela* subsp. *piscicida* from moribund sea bream.

No. of examined fish	Recovery rate	
	Positive	%
50	9	18.00

Table (2): Rate of isolation of Ph.damsela subsp. piscicida from the internal organs of moribund sea bream.

Examined organs	Positive results	
	No.	%
Kidneys	9	18.oo
Liver	6	12.00
Spleen	5	10.00
Intestinal contents	3	6.00
Overall total	23	11.50

Table (3): Mean agglutinating antibodies titres in vaccinated Mugil cephalus fish with chloroform killed cell bacterin of *Ph. damsela* subsp. *piscicida*.

2-weeks post-primary vaccination		2-weeks post-secondary vaccination	
Vaccinated fish	Control fish	Vaccinated fish	Control fish
37.3 ± 0.7	13.8 ± 0.244	459 ± 0.341	13.8 ± 0.244

Table (4): Results of experimental challenge on vaccinated and control Mugil cephalus fish using live *Ph. damsela* subsp. *piscicida* culture with determination of vaccine potency.

Marker of vaccination	Vaccinated group		Control group	
	No.	%	No.	%
Mortality rate	1	10.00	2	40.00
RPS	75 %			

RPS : Relative percent survival.

Table (5): Re-isolation of Ph. damsela subsp. piscicida from the internal organs of dead Mugil cephalus post-experimental challenge with live culture.

Examined organ	Vaccinated group(1)*	Control group(2)*
Liver	1	1
Spleen	1	2
Kidneys	0	2

* No. of dead Mugil cephalus post-challenge.

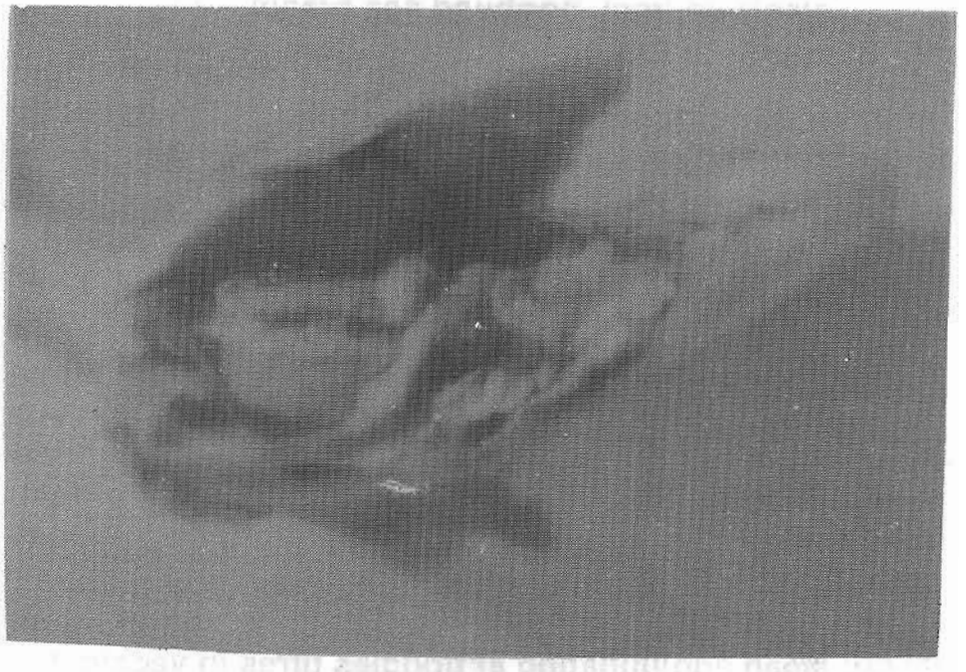

Figure (1): P.M. lesions in seabream fish infected with *Ph. damsela* subsp. *piscicida*.

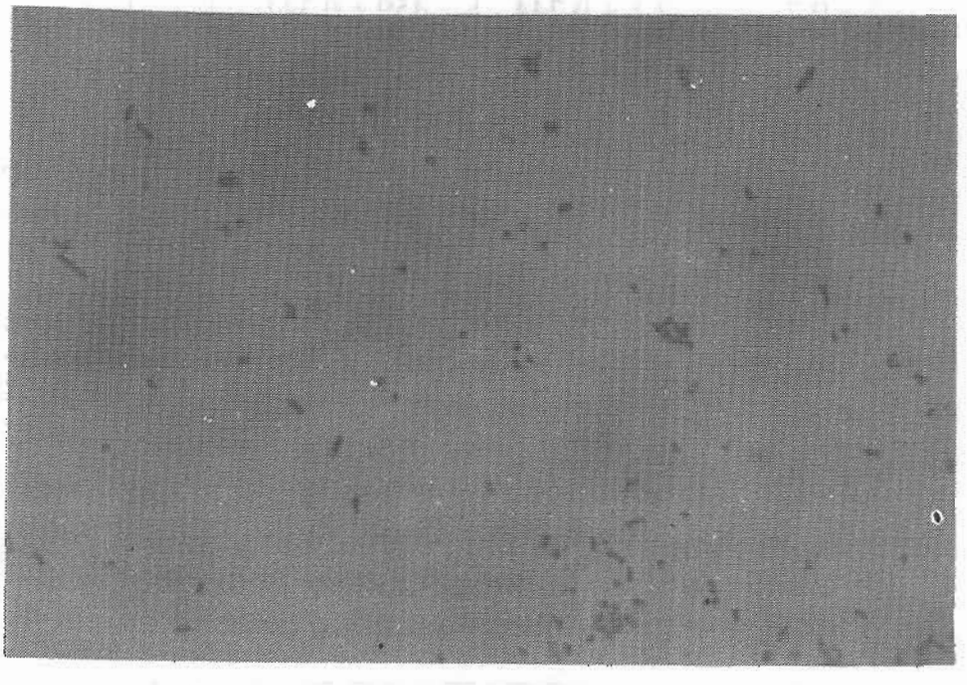

Figure (2): Morphological features of *Ph. damsela* subsp. *piscicida* in a bacterial film stained with Gram's stain.

الملخص العربي

محاولة لتحضير تحصين باستخدام الكلوروفورم ضد العدوى بميكروب الفوتوباكتريم دامسلا تحت نوع بسيسيدا المعزول من سمك الدنيس

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عزل ميكروب الفوتوباكتريم دامسلا تحت نوع بسيسيدا من سمك الدنيس المريض بمعدل ٠٠و١٨ % والذي كان عليه أعراض متمثلة في لون الجلد قاتم، استسقاء في التجويف البطني، تآكل في الذيل مع فقد القشور وكانت الصفة التشريحية متمثلة في شحوب الكبد مع تضخم المرارة و بثرات بيضاء مماثلة لبثرات السل في الكبد. كان معدل العزل عالي من الكلى ٠٠و١٨ % يليه الكبد ٠٠و١٢ % ثم الطحال ٠٠و١٠ % و محتويات الأمعاء ٠٠و٦ %. تم تحضير تحصين من هذا الميكروب بعد قتله بالكلوروفورم والذي اثبت كفاءته في تحصين سمك البوري ضد هذا المرض معطيا أجسام مناعية متلزنة بمعدل ٣و٣٧ ± ٧و. بعد التحصين الأولى و بمعدل ٤٥٩ ± ٣٤١و٠ بعد التحصين الثاني وقد بلغ معدل الحماية للأسماك المحصنة ٠٠و٧٥ %.