TRIALS TO CREATE EDWARDSIELLOSIS NATIVE VACCINES FOR FRESHWATER FISH IN EGYPT

EL-JAKEE, J. K. [1], , M.S. MARZOUK[1], N.A. MAHMOUD[2] AND MAHA, A. EL-HADY[2]

1. *Faculty of Veterinary Medicine, Cairo University*
2. *Fish Diseases Research Department, Animal Health Research Institute, Dokki, Giza.*

Abstract

In the present study *Edwardsiella tarda* isolated from local *Clarias gariepinus* was highly pathogenic and effective when experimentally inoculated in *Oreochromis niloticus* fish. So formalized inactivated bacterin, outer membrane protein and lipopolysaccharide (LPS) vaccines were prepared from this isolate and injected intraperitoneally (IP) in three groups of *Clarias gariepinus* fish. The quality control analysis proved that the prepared vaccines were free from any contaminant. The safety tests illustrated that the prepared vaccines did not show any abnormalities or adverse reactions among the injected fish during the observation days. Antibody titers to *Edwardsiella tarda* vaccines were estimated using microagglutination and ELISA methods. The agglutinating and ELISA antibody titers of fish vaccinated with outer membrane protein were 2560 and 2570 at 4 weeks post vaccination, respectively, followed by LPS (1280 and 2132) and formalin-inactivated vaccine (1040 and 1382), respectively. The laboratory challenge study showed that the protection rates of Outer membrane protein, formalin-inactivated and Lipopolysaccharide vaccines were 100%, 96% and 92%, respectively. SDS-PAGE profiles analysis of the prepared vaccines illustrated that, the sonicated *Edwardsiella tarda* cells had 18 bands (70.8, 66.6, 61.8, 56.7, 54.2, 51.5, 48.4, 43.9, 36.1, 31, 27.6, 22.7, 19.3, 17.4, 16.4, 14.9, 13.8 and 13 kDa. While the outer membrane protein of *Edwardsiella tarda* had ten protein bands of 55.9, 51.5, 39.8, 28.8, 24.2, 19.5, 17.1, 14.9, 13.3 and 13.1 kDa. Meanwhile, 6 bands (45.3, 35.4, 18.6, 15.5, 14.6 and 13.9) were extracted from LPS antigen of *E. tarda.*

INTRODUCTION

Infectious diseases are the main cause of economic losses in aquaculture industry which is negatively impacted by various pathogenic organisms (Plumb, 1997). *Edwardsiella tarda*, a facultative aerobic Gram negative enterobacterium of the family *Enterobacteriaceae*, is the causative agent of edwardsiellosis in freshwater and marine fish both in farmed and wild population allover the world. Abdel-Lah and Shamrukh (2001) detected Edwardsiella and other hydrogen sulfide-producing organisms among grounded water in a Nile Valley village, Egypt. Fifteen actinomycetes were isolated by Saleh *et al.* (2007) and screened for their antibacterial activity against E. coli and pathogenic E. tarda isolated from the Lake Bardawil, Egypt. Edwardsiellosis has been

recorded in a diverse array of commercially important fish including eels , channel catfish , mullet , Chinook salmon , flounder , carp , tilapia and striped bass (Thune *et al*.,1993) .Edwardsiellosis is a septicemic disease characterized by extensive lesions in the skin, muscle and internal organs . The use of antibiotics and chemotherapeutics with regards to their marginal effectiveness and high cost (Sealey and Gatlin, 2001) has achieved partial success, as they may cause accumulation in the environment and/or fish, thus posing potential threats to consumers and the environment. Although *Edwardsiella tarda* is considered as bacteria of fish, reptiles and other cold-blooded animals, it can infect mammals and humans, causing gastroenteritis, meningitis (Nettles and Sexton, 1997 and Pavanelli *et al*., 1998), nephritis (Pastor, 1981) and liver and skin abscesses (Noga, 1996 and Manchanda *et al*., 2006). Some vaccination methods have been attempted against edwardsiellosis through reviewing the update literature. The methods include immersion in a solution containing heat-killed cells or formalin-killed cells (FKC) of *Edwardsiella tarda* (Song and Kou, 1981), and intramuscular injection of the FKCs or crude lipopolysaccharide (LPS) of *Edwardsiella tarda* (Salati and Kusuda, 1985). *Edwardsiella tarda* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) may be an effective vaccine candidate against infection by *E. tarda* in Japanese flounder *Paralichthys olivaceus* (Liu *et al*., 2007). Protection of tilapia (*Oreochromis mosambicus*) against edwardsiellosis by vaccination with *Edwardsiella tarda* ghosts (Kwon *et al*., 2006). So the objectives of the present study was to compare the immunizing potency of 2 proteins extracted from local *E. tarda* isolate (outer membrane protein and LPS antigens) and a formalin inactivated prepared vaccine in groups of *Clarias gariepinus* to determine their effectiveness.

MATERIALS AND METHODS

1- Vaccinal strain

Edwardsiella tarda local isolate recovered from diseased *Clarias gariepinus* was identified by morphology, colonial characters and biochemical tests according to Quinn *et al*. (2002).

2- Pathogenicity of *Edwardsiella tarda* in *Oreochromis niloticus* fish

A total of 48 apparently healthy *Oreochromis niloticus* were purchased from Manial Sheha, Giza Governorate, with an average body weight 80.0±20.0 g, and transferred alive to Fish Diseases Department, Animal Health Research Institute, Dokki, Giza. Fish were divided into two equal groups each of 24 and kept in chlorine-free tap water in two full glass aquaria (120×60×40). Fish were acclimated to experimental conditions for 2 weeks with continuous aeration and the water temperature was thermostatically controlled and kept at 22±2˚C. Fish were randomly

screened for the presence of *Edwardsiella tarda* and were found to be free from the risk of natural infection and free from other pathogens. Each fish in the first group was inoculated intraperitoneally (IP) with 0.5 ml / fish of *Edwardsiella tarda* (10^6 CFU /ml) (Austin and Austin, 1993). However, fish in the second group was kept as a control and injected I / P with a dose of 0.5 ml / fish of sterile physiological saline. The two fish groups were kept under observation for three weeks to record the pathogenesis of the infected group.

3- Preparation of *Edwardsiella tarda* vaccines

I- Preparation of formalin- inactivated vaccine (FIV)

FIV was prepared by addition of formalin to the bacterial cell culture incubated at 25◦C with agitation for 36 hours to a concentration of 1% (Vinitnantharat and Plumb, 1992).

П- Preparation of outer membrane protein (OMP) vaccine

Edwardsiella tarda isolate was grown onto tryptic soy agar and harvested in HEPES buffer (PH 7.4). The bacterial cells were disrupted using a sonicator and centrifuged. The clear gel-like pellet was resuspended in 10 mM HEPES buffer to which an equal amount of 2% detergent solution (sodium sarcosinate) was added then left overnight at 4◦C, the detergent insoluble fraction was harvested by centrifugation at 100 000 xg/60 min. at 4◦C and the pellet was resuspended in distilled water and dialyzed for 24 hours (Newton *et al*., 1990). The protein concentration was determined by use of the Bradford protein assay method according to Bradford (1976).

Ш- Preparation of lipopolysaccharide (LPS) vaccine

Bacteria were washed with water and treated successively with ethanol, acetone and twice with ether. Extraction of LPS by using an extraction mixture containing liquid phenol (90 g phenol+ 11ml water), chloroform and petroleum ether in a volume ratio of 2: 5: 8 , respectively. Bacteria were placed in centrifuged vessel and extraction mixture was added in a ratio of 1:4. Then the procedures completed according to Gutierrez and Miyazaki, 1994.

4- Quality control of the prepared vaccines

The prepared vaccines were tested for purity, sterility and safety according to the Code of American Federal Regulation (1985).

5- Fish vaccination: (Vinitnantharat and Plumb, 1992 and Gutierrez and Miyazaki, 1994).

A total of 100 apparently healthy *Clarias gariepinus* obtained from Nile Delta were acclimated to experimental condition for 2 weeks. Fish were randomly screened

for the presence of *Edwardsiella tarda* and were found to be free from the risk of natural infection and free from other pathogens, then divided into four equal groups as following:

Group 1: Fish were vaccinated I/P with 0.1 ml of formalin- inactivated bacterin at a concentration of 2×10^8 formalin-inactivated cell /ml.

Group 2: Fish were vaccinated I/P with 0.1 ml /fish of the Outer membrane protein containing 1.5 mg total protein /ml.

Group 3: Fish were vaccinated I/P with 0.1 ml /fish of the Lipopolysaccharide vaccine containing 1 mg total protein /ml.

Group 4: Fish were kept as a control; each fish received 0.1 ml of sterile saline intraperitoneally.

- Two weeks post-vaccination each fish group received the same dose and concentration of the 1st dose as a booster dose.

6- Collection of blood samples

Blood samples were collected from fish pre-vaccination and weekly post-vaccination up to 9 weeks from both vaccinated and unvaccinated fish. The collected blood samples were allowed to clot overnight at 4◦C then centrifuged at 3000 xg for 10 min. The separated sera were stored at -20 ◦C till used.

7- Evaluation of humoral immune response among vaccinated fish by using

I- Enzyme Linked Immunosorbent Assay (ELISA):

It was carried out according to Klesius *et al*. (1991). Calculation of antibody titer was carried by the following equation according to Briggs and Skeeles (1984).

Log 10 titer = 1.09 (log 10 s/p) + 3.63

$$S/P = \frac{\text{Sample mean - Negative control}}{\text{Positive control - Negative control}}$$

П- Microagglutination test (MAt)

It was carried out according to Shelby *et al*. (2001) and antibody titers were expressed as geometric mean titer (GMT) according to Brugh (1977).

8- Challenge test

Two weeks after the booster dose, fish of all groups were challenged I/P with 0.5 ml culture suspension of 16 hours tryptic soy broth containing 10^6 CFU /ml of *Edwardsiella tarda* (Austin and Austin, 1993). All challenged fish (vaccinated and unvaccinated) were kept under observation for 4 weeks and the mortality rate was recorded.

9- Detection of protein polypeptide bands of the prepared vaccines through SDS- PAGE: According to Sambrook *et al*. (1989).

10- Statistical analysis:

The obtained results were analyzed by analysis of variance "LDS" test according to Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

In the present study experimental infection of *Oreochromis niloticus* fish with *Edwardsiella tarda (*0.5 ml / fish, 10^6 CFU /ml) *was* effective in producing mortality rate reached to 41.64%. The most common clinical disease manifestations were hemorrhages allover the fish body, skin darkening, pale skin areas with detached scales and hemorrhagic protruded vent. As the disease progressed, there was bilateral exophthalmia as well as abdominal dropsy. The post-mortem findings of the experimental inoculated fish with *Edwardsiella tarda* showed yellowish ascetic exudates filled the peritoneal cavity, which discharged at abdominal incision with a characteristic putrid odor. The spleen and liver were congested and enlarged, the intestine showed hemorrhagic enteritis. As the disease progressed, there was severe adhesion between the visceral organs and the viscera appeared as one homogenous mass with liver necrosis or paleness. The clinical signs of infection (photos,1&2) were similar to the results of Miwa and Mana (2000). Furthermore, Galal *et al.* (2005) added that, the clinical signs of experimentally infected *O.niloticus* showed loss of scales and coloration, as well as severe edematous swelling at the site of infection. After 2 days pos inoculation, the skin was raised forming hemorrhagic swelling and hemorrhagic exudates with offensive odor.

Although *Edwardsiella tarda* isolated from *Clarias gariepinus* it was highly pathogenic and effective when experimentally inoculated in *Oreochromis niloticus* fish as *Edwardsiella tarda* is considered a dangerous septicemic disease affecting different species of cultured fish and leading to high economic losses. In this concern Noga (1996) stated that *Edwardsiella tarda* was generally related to freshwater environments, moreover it can infect the skin or cause gastroenteritis or systemic infections to clinicians during a clinical work-up. Badran (1993) recorded an outbreak of edwardsiellosis among Nile tilapia reared in ponds supplied with domestic wastewater in Egypt. Catfish is a highly resistant species and considered a good source of large amount of blood opposite to O.niloticus. So, 3 vaccines (formalized inactivated, lipopolysaccharide and outer membrane protein vaccines) were prepared from *Edwardsiella tarda* isolated from local environment and injected intraperitoneally in 3 groups of catfish. Shoemaker and Klesius (1997) concluded that, oral bacterin failed to induce specific antibody production or provide protection. Previously Thune *et al.* (1993) suggested that the variability of the field trials with oral bacterin vaccines

was influenced by loss of antigen to the pond water. The intraperitoneal injection of inactivated whole bacteria was more consistent in inducing detectable titers (Ainsworth et al., 1995). Among formalized inactivated vaccine, the antibody titers were 1040 and 1382 at 4 weeks post vaccination by using microagglutination and ELISA methods, respectively (Table,1). Waterstrat et al. (1989) compared microtiter agglutination and ELISA for detection of channel catfish antibody to *Edwardsiella ictaluri*, and found strong correlation between the results of the two techniques, but the ELISA more sensitive. According to our results, the intraperitoneal administration of outer membrane vaccine caused a gradual increase in mean serum antibody titers over the sampling periods. However, there was a significant rise in mean antibody titers from days 21 to 28 sampling for outer membrane protein vaccine. Rapid enzyme-linked immunosorbent assays (ELISA) demonstrated that OMP produced a weak, but observable antibody response by 21 days post injection (Bader et al., 2004). In this concern, Ainsworth et al. (1995) concluded that the antibody responses in fish administered OMP orally were significantly less than in fish given intraperitoneal injections. They added that OMP would not enhance innate cellular components of immunity to *Edwardsiella* following oral vaccination. From the point of our work, vaccinated fish with lipopolysacchride showed increase in agglutinating and ELISA antibody titers in comparison to the controls. The highest titer was recorded by Salati *et al.* (1984) in eels immunized with crude LPS. The intramuscular injection of eels and red sea bream with *Edwardsiella tarda* LPS resulted in a demonstrable humoral immune response (titer = 1:2048) as recorded by Salati *et al.* (1987 a and b). The crude LPS induced better protection than highly purified preparation whereas the geometric mean of agglutinating antibody titer reached to 5043 and 2048., respectively (Ellis, 1988). Nile tilapia, *O. niloticus,* vaccinated with *Edwardsiella tarda* crude LPS and reared under optimal environmental conditions had good levels of secreted antibody in the body surface mucus appeared from first week post vaccination and gradually elevated in relation to time post vaccination (Badran, 1995). The only way of accurately measuring the potency of a vaccine against a pathogen is to determine, under controlled conditions, whether or not the vaccinated fish are protected against the infectious agent from which the vaccine is made. Ellis (1988) stated that potency of vaccines can only be tested in controlled laboratory conditions because the cause of mortality is not always known in the pond environment. The vaccinated and control groups were challenged intraperitoneally 14 days after booster vaccination with 10^6 CFU/ml of *Edwardsiella tarda*. Our laboratory challenged study showed that catfish which were vaccinated by formalin inactivated vaccine had protection rate of 96% as shown in Table (2). Vaccination with *Edwardsiella tarda*

formalin killed cells (FKCs) delayed mortality following experimental *Edwardsiella tarda* infections (Song *et al.*, 1982 and Salati and Kusuda, 1985). In those studies, vaccination with FKC was found to enhance phagocytic activity of Japanese eel leucocytes in vitro. Gutierrez and Miyazaki (1994) added that, vaccination with FKCs of *Edwardsiella tarda* resulted in a survival of 40% when challenge doses of *Edwardsiella tarda* did not exceed 10^5 CFU/fish. Meanwhile Shoemaker and Klesius (1997) recorded high mortalities in the formalin-killed bacterin vaccinates regardless of antibody titer. In the present study, the potency of the prepared *Edwardsiella tarda* outer membrane protein vaccine was measured by calculating the protection percent. No mortality could be detected among the vaccinated group. The mortality of fish vaccinated with LPS was 8% meanwhile the unvaccinated control group had 100% mortality these results indicated efficacy of the vaccine. In this concern Miyazaki *et al.* (1992) concluded that the survival of unvaccinated controls was 0-20% while vaccination with LPS of *Edwardsiella tarda* gave a level of protection that afforded almost 60% survival. The SDS PAGE analysis of sonicated *Edwardsiella tarda* cells used for coating ELISA plates revealed that 18 bands (70.8, 66.6, 61.8, 56.7, 54.2, 51.5, 48.4, 43.9, 36.1, 31, 27.6, 22.7, 19.3, 17.4, 16.4, 14.9, 13.8 and 13 kDa) could be detected as shown in Photo (3) and Table (3). The cell wall fraction contained 21 proteins from 97 to 8 kDa *(*Bader *et al.*, 2004). Outer membrane protein of *Edwardsiella tarda* isolate was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis as shown in Photo (4) and Table (3). The outer membrane protein of *Edwardsiella tarda* had ten protein bands of 55.9, 51.5, 39.8, 28.8, 24.2, 19.5, 17.1, 14.9, 13.3 and 13.1 kDa. Meanwhile, 6 bands (45.3 , 35.4 , 18.6 , 15.5 , 14.6 , 13.9) were extracted from LPS antigen of *E. tarda* as shown in photo (5) and Table (3) . Three different extraction methods were used by Tu and Kawai (1998) for preparing OMPs from the cell membrane fraction of *Edwardsiella tarda* strain. SDS extracted 3 major OMPs at 37, 40 and 43 kDa. A 37 kDa major outer membrane protein (MOMP) of *Edwardsiella tarda* EF-1 strain was purified by Tu and Kawai (1999). It was concluded that the MOMP was protective antigen of *Edwardsiella tarda* which had a potential to be used for developing vaccine against *Edwardsiella tarda*. In conclusion from the present trial it is clear that outer membrane protein vaccine was superior and gave the best results as ELISA and MAt mean antibody titers were higher than that of formalized inactivated vaccine and LPS vaccine. Also, it can offer 100% protection among the vaccinated fish. From the present study it could be concluded that *Edwardsiella tarda* vaccine application in pisciculture should be performed. Edwardsiellosis is a disease of fish culture, however, the disease has a wide range of hosts and as the culture of warm water species expands in tropics and subtropics so

the problem from edwardsiellosis may also expand. There is still much to be done concerning the feasibility of immunizing fish by immersion, the duration and specificity of the protection. Future work should be directed to the usefulness of OMP in fries and fingerlings.

Table 1. The mean antibody titers among the vaccinated fish groups measured by MAt and ELISA

Type of vaccine / serum / Date of collection / week	FIV		OMPV		LPSV		Unvaccinated Fish (control) group	
	MAt	ELISA	MAt	ELISA	MAt	ELISA	MAT	ELISA
0 week	0	0	0	0	0	0	0	0
1st week	160	992	680	1556	640	1288	0	32
2nd week *	320	1063	844	1663	788	1414	0	24
3rd week	640	1192	1280	2023	905	1893	0	40
4th week **	1040	1382	2560	2570	1280	2132	10	40
5th week	640	983	970	1877	905	1647	-	-
6th week	680	1184	1040	1893	970	1721	-	-
7th week	735	1267	1114	2023	1040	1829	-	-
8th week	788	1289	1194	2142	1114	1893	-	-
9th week	520	1012	640	1751	640	1040	-	

Mean ± SE	FIV	OMPV	LPSV	Control	LSD
ELISA	1151.56 ± 48.48 c	1944.22 ± 99.82 a	1650.78 ± 114.65 b	15.11 ± 6.17	229.98***
MAT	613.67 ± 86.08 b	1146.89 ± 191.02 a	920.22 ± 70.59 ab	1.11 ± 1.11	318.45***

*: Significant variation between groups by one ways ANOVA at P ≤ 0.05.
a-c = significance level
FIV: formalin-inactivated vaccine OMPV: outer membrane protein vaccine
LPSV: lipopolysaccharide vaccine * Booster dose ** Challen

Table . 2. Results of the challenge test among the vaccinated fish groups

Grouping	Total no. of fish	Mortality		Protection%
		No.	%	
FIV	25	1	4	96
OMPV	25	0	0	100
LPSV	25	2	8	92
UNV	25	25	100	0

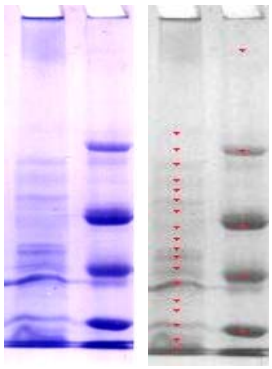
FIV: formalin-inactivated vaccine OMPV: outer membrane protein vaccine
LPSV: lipopolysaccharide vaccine UNV: Unvaccinated (control) group

Table . 3. The protein profile analysis of *Edwardsiella tarda* vaccinal antigens

Band number	Molecular weight / kDa			
	Marker	Sonicated Ag	OMP	LPS
1	97	70.8	55.9	45.3
2	66	66.6	51.5	35.4
3	45	61.8	39.8	18.6
4	20.1	56.7	28.8	15.5
5	14.4	54.2	24.2	14.6
6		51.5	19.5	13.9
7		48.4	17.1	
8		43.9	14.9	
9		36.1	13.3	
10		31	13.1	
11		27.6		
12		22.7		
13		19.3		
14		17.4		
15		16.4		
16		14.9		
17		13.8		
18		13		

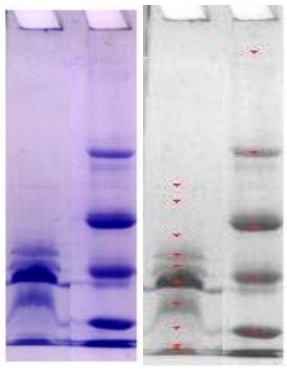
Photo. 1. *O.niloticus* fish injected I/P with *Edwaardsiella tarda* showing congestion allover the body surface, enlargement of abdomen and haemorrhagic

Photo.2. *O.niloticus* fish injected I/P with *Edwaardsiella tarda* showing adhesion between the visceral organs, severe haemorrhagic



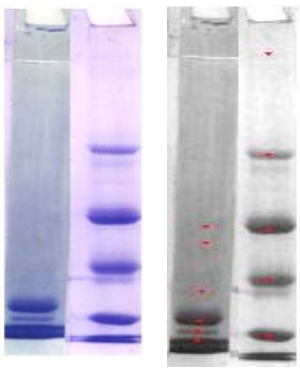
L M L M

Photo 3. SDS –PAGE analysis of Sonicated Edwardsiella tarda



L M L M

Photo 4. SDS –PAGE analysis of OMP of Edwardsiella tarda



L M L M

Photo 5. SDS – PAGE analysis of LPS of Edwardsiella tarda

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محاولات لتخليق لقاحات محلية لمرض الادواردسيلوزس لأسماك المياه العذبة فى مصر

جاكين كمال عبد الحليم الجاكي[١] ، محمد سيد محمد مرزوق[١] ، نشأت عبدالمتعال محمود[٢] ، مها عبد العظيم الهادي[٢]

١. *كلية الطب البيطري- جامعة القاهرة*
٢. *قسم بحوث أمراض الأسماك- معهد بحوث صحة الحيوان – الدقي – الجيزة - مصر*

فى هذه الدراسة ثبت ان الادواردسيلا تاردا المعزولة من اسماك دلتا النيل شديدة الضراوة و ممرضة عند حقنها فى اسماك البلطى النيلى. لذلك تم تحضير ثلاثة لقاحات مختلفة للادواردسيلا تاردا وهى الادواردسيلا تاردا المثبطة بمادة الفورمالين و بروتين الجدار الخارجى والليبوبولى سكاريد، وقد تم تحصين مجموعات من اسماك القرموط الافريقى باللقاحات المحضرة ، عن طريق الحقن البريتونى. وبإجراء اختبارات الجودة للقاحات المحضرة فقد تبين خلوها من أي تلوث (بكتريا هوائية ، بكتريا لاهوائية ، فطريات ، ميكوبلازما) و انها آمنة الاستعمال بالنسبة للأسماك. وبتقييم مستوى الأجسام المناعية فى مصل الأسماك المحصنة باللقاحات المختلفة و الأسماك غير المحصنة عن طريق اختبار التلازن العيارى و اختبار الاليزا. كانت نسبة الأجسام المناعية التى تم تقييمها عن طريق التلازن العيارى و الاليزا للأسماك المحصنة بلقاح بروتين الجدار الخارجى هى ٢٥٦٠ و ٢٥٧٠ فى الأسبوع الرابع بعد التحصين على التوالى. يليه الليبوبولى سكاريد (١٢٨٠ و ٢١٣٢) ثم الادواردسيلا المثبطة بمادة الفورمالين (١٠٤٠ و ١٣٨٢) على التوالى. وبإجراء اختبار التحدى فقد تبين أن مستوى الحماية ضد الميكروب كانت بنسبة ١٠٠% و ٩٦% و٩٢% فى الأسماك المحصنة بلقاح بروتين الجدار الخارجى و الادواردسيلا المثبطة بمادة الفورمالين و الليبوبولى سكاريد على التوالى . وتم أيضا إجراء التحليل الكهربائى اس دى اس للقاحات المحضرة.