

Study of Toxicity, Immunological and some Cytopathic Effects of *Vibrio cholerae* supernatant

**دراسة التأثيرات السمية والمناعية وبعض التأثيرات المرضية في عالق بكتريا
*Vibrio cholerae***

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Abstract:

Crude *Vibrio cholerae* extract was produced by centrifugation and filtration then the amount of protein was estimated by Folin- lowry method and was found equal to 550 microgram/ml. Extract activity was set by infant suckling mouse method. Toxicity was determined by measuring LD₅₀ and was found equals to 57microgram/ml. Rabbit Anti-bacterial extract antibodies was titrated by precipitation method and the result showed that the titer is 40. Ouchterlony double immunodiffusion test between several dilutions of the crude bacterial extract and rabbit antiserum showed positive results in 1/40 and 1/80 of the extract dilutions. The cytopathic effect of extract on mice hearts was also studied by injecting number of mice with 55 and 27.5 microgram/ml concentration of the crude extract and it showed that it has no certain effect on this organ.

Keywords: *Vibrio cholerae*, bacterial extraction, cytopathic effects.

المستخلص:

انتج المستخلص الزرعي الخام من بكتريا *Vibrio cholerae* بالطرد المركزي والترشيح ثم قدرت كمية البروتين باستخدام طريقة فون- لوري و وجد انها تساوي 550 مايكروغرام/مل، استخدمت طريقة الفأر الرضيع في تحديد فعالية السمية للمستخلص ، حُددت الفعالية السمية للمستخلص الخام بقياس متوسط الجرعة القاتلة و وجد انه يساوي 57 مايكروغرام لكل مل، تمت معايرة الأجسام المضادة للمستخلص في الارنب بطريقة الترسيب واطهرت النتائج ان العيار يساوي 40، كما اظهر اختبار الانتشار المناعي المزدوج في الهلام بين عدة تخافيف من المستخلص ومصل الارنب نتائج ايجابية للتخفيف 40/1 و 80/1، تضمنت الدراسة ايضا تحري الإعتلال الخلوي في قلوب الفئران بحقن عدد منهم بتركيزين هما 27,5 و 55 مايكروغرام/مل من المستخلص الخام و قد اظهرت النتائج عدم وجود تأثير يذكر في المقاطع النسيجية للعضو المذكور.

Introduction

Vibrio cholerae a member of the family Vibrionaceae is a facultatively anaerobic, Gram-negative, motile, non spore forming curved rod, about 1.4-2.6 μm long, capable of both respiratory and fermentative metabolism (1). The bacterium is oxidase positive, reduces nitrate and is motile by means of a polar flagellum (2, 3, 4). Strains of *V. cholerae* and *V. mimicus* can grow in the absence of NaCl, a feature which distinguish these bacteria from other vibrios (5). *V. cholerae* is facultative human pathogen, can survive and thrive in aquatic environment, more than 200 serogroup (O1-O200) exist (6) but only toxigenic strains of the serogroup O1 and O139 cause cholera (7) which is an acute toxigenic diarrheal disease that three millions person have every year worldwide (8). Infection results from oral ingestion of the pathogen, the infective dose must be large ($\geq 10^8$), since many vibrios are killed by the hydrochloric acid in gastric juice (9). The symptoms of cholera include profuse watery diarrhea accompanied by abdominal pain, vomiting, fever, hypovoluminous shock and acidosis (10). The common virulence mechanism of the toxigenic strains is well characterized and depends on elaboration of the toxin coregulated pilus (TCP) and cholera toxin (CT) (11).

Non- O1, O139 serogroups are indigenous member of the aquatic ecosystem and are generally non pathogenic (12). However, the incidence of cholera outbreaks with serogroups other than O1/O139

has also been recorded, as these strains responsible for the sporadic outbreaks and have virulent factors but differs than those in epidemic O1 and O139 strains (13,14) including cholera like enterotoxin, El Tor hemolysin thermostable direct hemolysin (8), Shiga like toxin, Cytolysin, heat stable enterotoxin (NAG-ST), new cholera toxin (CT), a non membrane –damaging cytotoxin (NMDCY), hemagglutinin protease and serine protease of which few or all had been reported playing a role in clinical manifestation (10,15) Yet, non O1/O139 strains with *ctxA* and *ctxB* genes also have been observed (16, 17).

Observations showed that *Vibrio cholerae* O1infection induces an increase in inflammatory cells (18). Subsequent studies showed that *Vibrio cholerae* may induce increasing in innate factors including myeloperoxidase, lactoferrin, nitric oxide metabolites and eicosanoids. Natural cholera infection or cholera toxin induces a Th2-type of cytokine response resulting in an increase in specific IgG and IgE isotypes of antibodies and also increases in Interleukin (IL)-6 productions by mast cells suggesting involvement of innate cells in the immune response (19). This means *Vibrio cholerae* extract has both cytotoxic and immunological effects. Thus, our study was designated to study these approaches.

Materials and methods

V.cholerae (NAG) isolate was obtained from the central health laboratory in Baghdad which was diagnosed previously and re-diagnosed by inoculation on TCBS then identified by Api20E kit (20). Crude bacterial extract of *V.cholerae* was produced by inoculation 500 ml of brain heart infusion broth with *V.cholerae* isolate. After incubation for 24 hrs at 37°C, the bacterial extract was harvested by cooling centrifuge at 6000 r.p.m. for 30 minutes then filtrated by use of Millipore filter (22µm) (21).

The Extract activity was measured by infants suckling mouse method (22). Amount of protein was determined by lowry method (23).

Two types of experimental animals were used in this research, mice and rabbits. 12 male mice (six weeks old) were divided into six groups for LD₅₀ experiment (group1) and other eight male mice of six weeks old were classified into three groups for studying the histopathological effects (group2). Two male rabbits were used for the serological tests (group3).

Five pairs of 10 male mice in group1 were injected intraperitoneally with 0.5 ml of the crude extract and from each two fold dilution prepared (1/10,1/20,1/40,1/80,1/160), using normal saline for dilution, the other two mice were injected with 0.5 ml of normal saline as a control. After five days all the injected mice were examined and the concentration which killed half of animals was determined and considered as LD₅₀.

Two subgroups (three mice each) of six mice in group2 were injected intraperitoneally with 0.5 ml of the crude extract and from each two fold dilution prepared from this stock (1/10,1/20) which represent 55 and 27.5 µg/ml concentration of the extract and less than LD₅₀ value to ensure survive of animals, using normal saline for dilution, the other two mice were injected with 0.5 ml normal saline as a control .After 5 days, all mice which injected were examined , The survive mice were killed, and dissected taking heart for study of histopathological changes. The histological sections were made (24).

The organs were fixed by 10% formalin (10 ml formalin + 90 ml 0.9% NaCl), then washed by tap water for several min. passing through a serial concentrations of alcohol (50%, 70%, 80%, 90% and 100%) for 2 hr. in each concentration, then cleared by xylol, saturated with paraffin at 60C° for 3 hr., embedded in pure paraffin; the blocks were cut into sections with 5 µm in thickness by using microtome. These sections were held on glass slides using Myer's albumin; they were left for drying at 37°C. Haematoxylin stain was used for 5-10 min., washed by tap water then with acidic alcohol then washed by tap water. After that Eosin stain was used for 15-30 sec. and then washed by D.W. Serial concentrations of alcohol were then used (70%, 90%, and 100%) for 2 min. in each concentration, cleared by xylol for 10 min.; then Canada balsam was used, covered by cover slide and examined by light microscope.

Group3 of rabbits were used to study the titer of anti-bacterial extract antibodies, one of them was injected intravenously with 1ml of the crude extract while the other was injected with physiological saline and considered as control, the animals were injected four doses in day 1,6 and 10 and 14, after two weeks of the last injection blood was obtained by heart puncture and serum was separated and kept at -20°C until been used.

Results and discussion

The result identified the isolate as *V.cholerae* where the colonies appeared yellow on TCBS agar, and the result of Api20E fulfilled this identification (25).

Crude *Vibrio cholerae* was extracted by filtration and crude extract activity was determined where amount of protein was equal to 550 µg /ml, toxicity of the crude extract was determined by LD₅₀ value as it was found equal to 57 µg/ml (Fig 1). This value reflects the severity of *V. cholerae* since low concentration of the extract caused killing for half of mice.

Titer of antibodies was equal to 40 which means reverse of the highest dilution that gives clear precipitation reaction. In previous study it was found that *Vibrio cholerae* enterotoxin induces enhanced antigen presentation by various antigen presenting cells, promote isotype differentiation in B cells leading to increased IgA formation and exert complex stimulatory as well as inhibitory effects on T cell proliferation and cytokine production (26).

Immunodiffusion test carried out between the bacterial extract of *Vibrio cholerae* as an antigen and the polyclonal antibody gave a single well-defined precipitin band showing a reaction of complete identity these investigation means that the the extract inclusions as enterotoxin was a good immunogenic and induced humoral immune response (27).

Ouchterlony test between the rabbits that injected with the crude extract antiserum and four dilutions of extract solutions (1/20, 1/40, 1/80 and 1/160) showed positive results and precipitation lines were found both in 1/40 and 1/80 dilutions of *Vibrio cholerae* crude extract (Fig 2), these multiple precipitation lines may be attributed to the mixture of antigens that crude bacterial solution may contain, where control showed no result.

These results support the hypotheses concerning *Vibrio cholerae* effects on the immune system and the role of cholera toxin on the adaptive immunity such as the increase of certain specific antibodies concentrations (28, 29, 30). In addition to its influence on the innate immune system which may be needed for the host to mount appropriate adaptive immune response to an infection (31). Such effects may need to be simulated in a vaccine to achieve long lasting protection from cholera (19, 32, 33).

A section of mice heart tissue which were injected intraperitoneally with the crude bacterial extract was made, it was found that no effect on heart tissue of both concentrations when compared with control mice heart tissue, (fig 3,4). Previous studies have shown that cholera enterotoxin, even in its purified form, affects the function of a wide variety of tissues. Although these effects contribute little, if any, to the disease state seen in human cholera, their study is of importance in understanding the cellular mechanism of action of cholera enterotoxin and in providing isolated cell systems in which to study this effect (34). Through this study, It should be mentioned that administration of *Vibrio cholera* crude extract intravenously to rabbit lead to the formation of obvious footpad edema which indicates the potency to affect non intestinal tissues (35). It was first noted that cholera stool infiltrates injected intracutaneously in rabbits caused an increase in skin permeability to protein, and that this effect also produced in rabbits and guinea pigs by cell-free infiltrates of *Vibrio cholerae* culture and the effect is neutralized by convalescent sera from cholera patients (36). In addition, previous study found that injection of cholera enterotoxin into the foot of rat produces after 2- to 4- hr delay, a prolonged but reversible local edema and the severity of which is dose dependent, similar changes also occur in the mouse (41 34).

Consequently, in a recent study (37) it was found that cholera toxin caused hydrobic to moderate degeneration of hepatocyte, slightly loosed of the tissue architecture with dilation of some sinusoids, where in the adrenal gland tissue it caused flattening of the lining cells with sluffing and

detached in capsule. Big vacule appeared with hydrobic degeneration of the zona tissue. Medulla suffered from hydrobic degeneration, congestion of blood vessel with haemorrhage in some area, while spleen tissue apparently not changed.

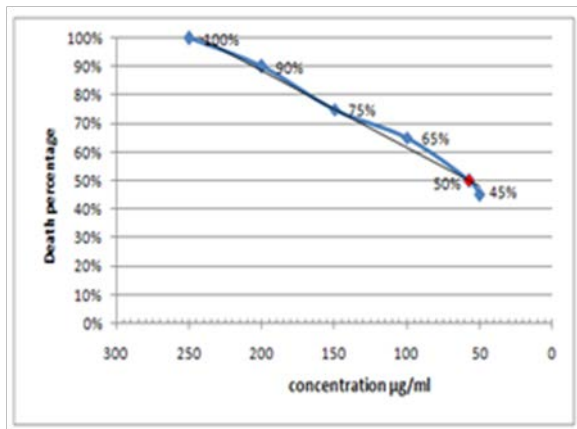


Fig1: Crude extract concentration and the corresponding death percentage where LD_{50} was $57\mu\text{g/ml}$.

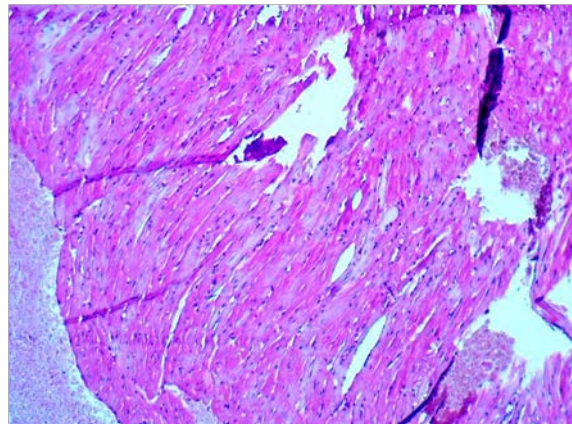


Fig3: No certain cytopathic effect on mouse heart tissue (1/10) dilution, (40X)

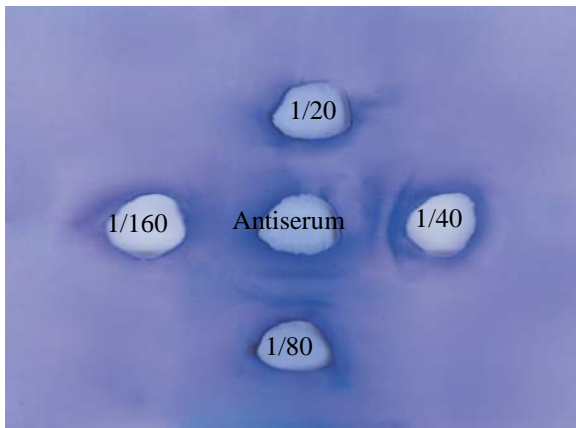


Fig2: Ouchterlony test and precipitation lines 1/40 and 1/80 dilutions of crude extract

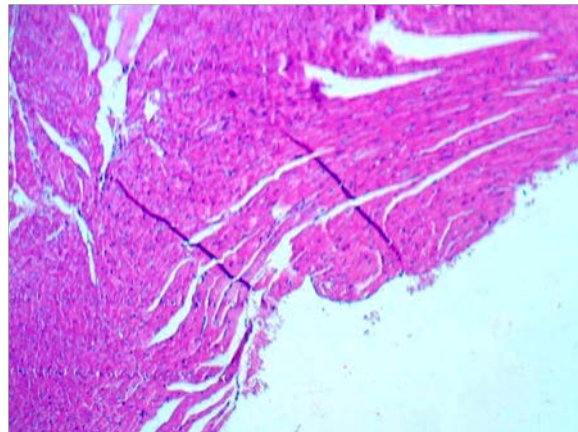


Fig4: Showing no certain cytopathic effect on mouse heart tissue (1/20) dilution, (40X)

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