Study the Ability of Enterotoxigenic *E.coli* Antigens To Induce Cellular Immune Response by Erythrocyte-Rosette Formation and Leukocyte Migration Inhibition Factor in Rabbits

دراسة قابلية مستضدات بكتريا الأشريكيا القولونية المنتجة للسموم المعوية على تحفيز الإستجابة المناعية الخلوية بواسطة التشكيل الزهري التائي و معامل تثبيط هجرة الخلايا البيض في الأرانب

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الخلاصة

هدف البحث: تهدف هذه الدراسة لمعرفة قابلية بكتريا Enterotoxigenic *E.coli* وسمومها المعوية على تحفيز الاستجابة المناعية الخلوية من خلال اختباري التشكيل الزهري التائي ومعامل تثبيط هجرة الخلايا البيض.

المنهجية: تم جمع ٣٠ عينة براز (stool) من المرضى المصابين بالإسهال الحاد المراجعين لمستشفى الصدر التعليمي في النجف الأشرف، في الفترة الممتدة بين شهري نيسان وحزيران ٢٠١٣ شخصت العزلات وفق الاختبارات المظهرية والكيموحيوية وباستعمال نظام API 20 E. تم التأكد من قابليتها على إنتاج السموم المعوية من خلال اختبار الفأر الرضيع Suckling Mice Assay (SMA) لجميع العزلات للتحري عن السم المعوي الثابت بالحرارة. حيث تم تحضير ثلاث أنواع من الانتيجينات (البكتريا المقتولة بالحرارة، والسم المعوي الخام، والسم المعوي الثابت بالحرارة المنقى جزئياً ETECST) والتي تم اختبارها في الأرانب المختبرية على المستوى الجهازي(الدم)والأنسجة المخاطية(الزائدة الدودية والاثني عشري).واجري التحليل الإحصائي باختبار اقل فرق معنوي.

النتائج: كانت نسبة بكتريا القولون المنتجة للسموم المعوية ٢٠% وبواقع ٦عزلات فيما بينت نتائج التجارب المناعية ان هناك زيادة معنوية في نسبة التشكيل الزهري التائي عند التجريع بالبكتريا المقتولة بالحرارة(٥٠.١%، ٤٩.٧% و ٥٣.٠%) مقارنة بمعاملة السيطرة (٢٣.١% ، ٣١.٨% و ٢٩.٦%) في الدم والزائدة الدودية والاثنى عشري على التوالي بينما كانت أعلى نسبة عند التجريع بالسم المعوي الخام و السم المعوي الثابت بالحرارة المنقى جزئيا في الأنسجة المخاطية للزائدة الدودية والتي كانت على التوالي (٤١.٣%) (٤٧.٤%).أما اختبار تثبيط هجرة الخلايا البلعمية فقد بينت الدراسة ان اكبر نسبة تثبيط كانت عند استخدام السم المعوي الثابت بالحرارة المنقى جزئيا (٥٤.٥%، ٤٧.٦% و ٤٣.٣%) في الدم والأنسجة المخاطية (الزائدة الدودية والأثني عشري) مقارنة بمعاملة السيطرة(82.0%، 79.5% و 79.5%).

الاستنتاج : للسموم المعوية الخام والمنقاة جزئياً قابلية جيدة لتحفيز الأستجابة المناعية الخلوية في الأنسجة المخاطية والدم.

التوصيات: محاولة تحضير جزيء حامل للسم المعوي المنقى جزيئاً تمهيداً لاستخدامه كلقاح ضد بكتريا Enterotoxigenic *E.coli*.

مفردات البحث: السم المعوي الثابت بالحرارة، السم المعوي الخام، التشكيل الزهري التائي ومعامل تثبيط هجرة الخلايا البيض .

Abstract:

Objectives :This study was aimed to investigate the ability of Enterotoxigenic *E.coli* and their enterotoxins to induce cellular immune response by means of erythrocyte-rosette formation and leukocyte migration inhibition factor tests.

Methods: Total of 30 stool sample was collected from patient with severe diarrhea whom attended to Al-Sadder teaching hospital in Al-Najaf city in period extended from April to June 2013. All bacterial isolates was identified according to morphological and biochemical testes and by API 20 E system, which further conformational tested for heat stable enterotoxin production by Suckling Mice Assay (SMA). three antigens (heat killed bacterial cell, crude enterotoxin and purified heat stable enterotoxin ETECST) was prepared and injected in experimental animals(rabbit) and tested in the level of systemic and mucosa (appendix and duodenum). Statistical analysis was done by least significant difference (LSD).

Results: The results showed that 20% (6 isolates) was Enterotoxigenic *E.coli* and the immune experiments showed significant increase level of E-rosette formation in rabbit injected with heat killed antigen (50.1%, 49.7% and 53.0%) compared with control (23.1%, 31.8% and 29.6%) in blood appendix and duodenum consecutively, while high level appeared in crude enterotoxin and ETECST in appendix (41.3%) and (47.4%) consecutively. The assessment of ETECST LIF in migration percent to sensitizer was showing high significant LIF (54.5%, 47.6% and 43.3%) compared with control (82.0%, 79.5% and 79.5%) in blood appendix and duodenum consecutively.

Conclusions: crud and partially purified enterotoxins have ability to induce cellular immune response in blood and mucosa.

Recommendation: prepare a carrier molecule model for partially purified enterotoxins to make a vaccine against Enterotoxigenic *E.coli* .

Key words: Enterotoxigenic E.coli, crudenterotoxin, partially purified enterotoxins erythrocyte-rosette formation and leukocyte migration inhibition factor.

INTRODUCTION:

Since Enterotoxigenic *E.coli* is still the most common cause of diarrhoea in the developing world resulting in approximately 20 percent of all diarrhoeal episodes in children in these areas, and the most frequent cause of diarrhoea in travelers [(1)].

strains ETEC colonize the small intestine, where they produce one or both of two enterotoxins called heat labile (LT) and heat stable (ST). Both toxins act by changing the net fluid transport activity in the gut from absorption to secretion. LT is structurally similar to cholera toxin and activates the adenylate cyclase–cyclic adenosine monophosphate system, whereas ST works on guanylate cyclase. The intestinal mucosa is not visibly damaged, the watery stool does not contain white or red blood cells, and no inflammatory process occurs in the gut wall. Gut cells activated by LT or cholera toxin remain in that state until they die, whereas the effects of ST on guanylate cyclase are turned off when the toxin is washed away from the cell[(2)].

The immunity in the small intestine was of prime importance for preventing disease. Hence, efforts in our laboratory to develop effective vaccines against cholera and ETEC diarrhoea have been focused on the identification of major protective antigens preventing binding of the bacteria in the intestine, suitable toxoids and optimal ways of inducing intestinal immune responses.Hence, immune responses to ST are not induced after infection with ST producing ETEC[(3)].

Heat-stable enterotoxins are low-molecular-weight, heat-stable, none or weak antigen proteins which do not cause intestinal secretion by activation of adenylate cyclase. Based on the detailed elucidation of the pathogenic mechanisms of cholera and ETEC, the protection against the causative organisms should be directed not only against colonization of the bacteria, but also against the toxin action. At least two types have been described, one with biological activity in suckling mice and piglets (STa, or named as STh, or ST-I) due to stimulation of particulate intestinal guanylate cyclase and a second which induces secretion by an unknown mechanism only in piglets (STb, or known as STp, or ST-II). Two partially cross-reacting antigenic variants of plasmid-coded LT, designated LTh and LTp, have been described in E. coli. LTh is associated with E. coli isolates from humans, and LTp is associated with E. coli isolates from pigs. The LT family from restricted geographical region exhibited a segregated pattern of dissemination that was revealed by a restriction enzyme site polymorphism[(4)].

LT from E. coli is a protein of approximately 86,000 daltons that consists of one A polypeptide and five B polypeptides held together by noncovalent bonds.LT is closely related to cholera enterotoxin (CT) in structure, antigenicity, and mode of action. Both LT and CT bind to ganglioside GM1 receptors on eukaryotic target cells via their B subunits. The pathway used by LT within the host cell is similar to that used by other AB5 toxins like CT [(5).]

The aim of study is to investigate the ability of Enterotoxigenic *E.coli* and their enterotoxins to induce cellular immune response and achieved by the following objectives:

1-Erythrocyte-rosette formation test.

2- leukocyte migration inhibition factor test.

METHODOLOGY:

Isolation and Identification: Total of 30 stool sample was collected from patient with severe diarrhea whom attended to Al-Sadder teaching hospital in Al-Najaf city Identification of *E.coli*

isolates were to the level of species using the traditional morphological and biochemical tests, according to the methods of [6][7].all isolates were confirmed identification with API 20 E system.

Experimental animals: New born albino mice (1-4) days used in Suckling mice assay (SMT). Wild type rabbit (1.5-2) kg used in immune experiment.

Enterotoxin Assays: Suckling Mice Assay (SMA)Test using infant mice is a convenient assay for ST enterotoxin. Supernatants of cultures (0.1 ml) injected with hypodermic needle (no. 30) into the milk-filled stomachs of infant mice (1-4) day old and weigh (0.9-1.3) g, the fluid accumulation in the intestine was measured after 4 h by determining the ratio of intestine to whole body weight. Usually two drops of a 2% solution of food dye were added to each 1 ml of inoculums. A ratio ≥ 0.08 was considered positive for heat stable enterotoxin[6].

Enterotoxigenic *E.coli* Partially purification of heat stable enterotoxin: The Sephacryl S-1000 gel was prepared by homogenizing it with Tris-HCl then settled and excess of buffer was removed .This step was repeated several times The gel was carefully poured into column (0.7×50) cm, down the wall to avoid accumulation of air bubble into it, the gel was left for few time to settle, the column outlet was opened .The packing continued to reach the length 36 cm. Later the packing gel was equilibrated with tris buffer pH 8. Using a flow rate of 3ml/45min , 3ml for each fraction were collected by using fraction collector and the absorbencies were measured at 280nm by spectrometer, and the peak fraction that showed enterotoxin activity was collected and concentrated by dialysis against poly ethylene glycol for further tests[7].

Heat killed bacterial Antigen:was prepared according to[8].

Immunization Protocol for Crude Enterotoxin and ETECST: Three adult rabbits were immunized intramuscularly with 100 µg of crude enterotoxin and another three rabbits with **ETECST**, for each of the two groups an equal volume of Freund's incomplete adjuvant were used. Two booster injections were given at weekly intervals. In the fifth week the animal was sacrificed after anesthetized by chloroform[9]. Then blood and mucosal sampling were done according to the following:

Systemic Blood Sampling : From each rabbit, a blood sample of 7-8 ml was drawn aseptically by heart puncture in a disposable syringe. 2.5ml of the blood sample were left at room temperature till being clotted, and then centrifuged at 3000 rpm for 5min. The sera were aspirated from whole blood and saved in test tube at -20°C till testing time[4].

Enteromucosal Sampling (Appendix and Duodenum): From each rabbit, 10-12cm from appendix and duodenum were collected in an aseptically method. The appendix and duodenum were opened by using sterile and clean scissor. The digested material was removed by washing them with normal saline. The mucosa and submucosa were scraped by sterile surgical scalpel and placed in another sterile petridishe containing 10 ml of normal saline. By sterile pasture pipette the suspension was transferred to sterile plastic test tube and shacked by vortex for 5min. The suspension was centrifuged at 4000 rpm for 20min. supernatant was ignored and the precipitate was washed three times with normal saline and preserved in at ratio 1:1 in Alsever's solution for studying NBT test [7].

Lymphocyte Suspension Preparation: Two ml of heparinized blood or mucosal suspension was gently mixed with 4ml of Hepes-BSS. The diluted blood was carefully added to the 2ml of lymphoprep, alongside the inner edge of the tube.The mixture above was centrifuged at 4000rpm for 15min.The leukocytes at the interface were aspirated by pasture pipette and washed twice in Hepes-BSS then suspended in the same buffer[4].

Erythrocyte-rosette formation Test: The E-rosette test was done according to the method of [10]. Sheep red blood cells (sRBC) was stored at 4°C in Alsever's solution and used for 1 week. Before use, the cell were washed twice and adjusted to a 5% suspension in N.S. The sRBC cell

suspension (0.25ml) was mixed with (0.25 ml) of lymphocyte suspension and incubated at 37°C for 15min. The mixed cell suspension was spun at 1000 rpm for 5min. and then incubated overnight at 4°C. The supernatant fluid was removed, and the top layer of the pellet was gently resuspended by shaking. One drop of the cell suspension was mounted on to a glass slide, covered by cover slip. Two hundred lymphocytes were counted, and all of the lymphocytes binding more than 3 sRBC were considered positive. Same method was used to detect E-rosette formation of mucosal lymphocytes.

Leukocyte Migration Inhibition Factor (LIF): Measurement of migration inhibition factor was done according [11], as follow: Agar-A medium was prepared in a sterile plastic plate (2%agar), and two well were done with 2cm in diameter on plate of agar. Capillary tubes filled with systemic blood or mucosal suspension was put in each well after centrifugation by hematocrite centrifuge for 10 min. Eagle basal medium (0.1ml) was put in each well; one of these wells was considered as control. 0.1 ml of each *E.coli* antigens prepared in this study was added in one well. Incubate at 37°C for 24hr in humid environment. Measurements of LIF by oculometer, same steps were used for control and normal saline was added instead of antigen sample. LIF was measured from the following equation:

$$LIF = \frac{Distance\ with\ sensitizer}{Distance\ without\ sensitizer} \times 100$$

Statistical Analysis : Analysis of variance (ANOVA) and the least significant difference (LSD) were used for the statistical analysis of the results and P-values at levels ($P<0.05$) were considered to be statistically significant. These calculations were carried out according to program SPSS, version 14.

RESULTS:

Erythrocyte-rosette formation test (E-rosette test):

Table (1) E-rosette test for Enterotoxigenic *E.coli* antigens on systemic cellular immune response in Rabbit

Antigen	Mean ± SD
Killed antigen	50.1 ± 1.9
Crude enterotoxin	31.7 ± 1.4
ETECST	39.6 ± 1.8
Control	23.1 ± 1.9

LSD (0.05) = 1.729

Table (1) show significant increase level of killed antigen in E-rosette test (50.1) compared with control treatment (23.1) on systemic cellular immune response.

Table (2) E-rosette test for Enterotoxigenic *E.coli* antigens on mucosal (Appendix) cellular immune response in Rabbit

Antigen	Mean ± SD
Killed antigen	49.7 ± 1.3
Crude enterotoxin	41.3 ± 0.8
ETECST	47.4 ± 1.4
Control	31.8 ± 1.5

LSD (0.05) = 1.258

Table (2) show significant increase level of ETECST in E-rosette test (47.4) compared with control treatment (31.8) on mucosal (Appendix) cellular immune response

Table (3) E-rosette test for Enterotoxigenic *E.coli* antigens on mucosal (Duodenum) cellular immune response in Rabbit

Antigen	Mean ± SD
Killed antigen	53.0 ± 1.6
Crude enterotoxin	39.3 ± 2.0
ETECST	44.0 ± 1.8
Control	29.6 ± 1.8

LSD (0.05) = 1.751

Table (3) show significant increase level of Killed antigen in E-rosette test (53.0) compared with control treatment (29.6) on mucosal (Duodenum) cellular immune response.

The formation of E-rosette in mucosal sample is higher than of systemic except for systemic killed antigen (50.1) which higher than of appendix (49.7) and lower than of duodenum (53.0).while the partially purified enterotoxin show the high level of E-rosette formation in appendix (47.4) then in duodenum (44.4) and in systemic (39.6). The crud enterotoxin showed low level of e-rosette formation (31.7, 41.3 and 39.3) in systemic, appendix and duodenum consecutively. All result were statistically significant at ($p<0.05$) compared with control (23.1, 31.8 and 29.6) in systemic, appendix and duodenum consecutively, tables (1), (2) and (3).

Leukocyte migration inhibitory factor (LIF) test:

Table (4) LIF test for Enterotoxigenic *E.coli* antigens on systemic cellular immune response in Rabbit

Antigen	Mean ± SD
Killed antigen	71.3 ± 1.7
Crude enterotoxin	66.1 ± 1.3
ETECST	54.5 ± 1.8
Control	82.0 ± 1.7

LSD (0.05) = 2.044

Table (4) show significant increase level of ETECST in LIF test (54.5) compared with control treatment (82.0) on systemic cellular immune response.

Table (5) LIF test for Enterotoxigenic *E.coli* antigens on mucosal (Appendix) cellular immune response in Rabbit

Antigen	Mean ± SD

Killed antigen	57.5 ± 0.5
Crude enterotoxin	52.6 ± 0.8
ETECST	47.6 ± 0.8
Control	79.5 ± 3.0

LSD (0.05) = 1.972

Table (5) show significant increase level of ETECST in LIF test (47.6) compared with control treatment (79.5) on mucosal (Appendix) cellular immune response.

Table (6) LIF test for Enterotoxigenic *E.coli* antigens on mucosal (Duodenum) cellular immune response in Rabbit

Antigen	Mean ± SD
Killed antigen	56.1 ± 1.1
Crude enterotoxin	54.6 ± 1.5
ETECST	43.3 ± 1.6
Control	79.5 ± 2.9

LSD (0.05) = 2.332

Table (6) show significant increase level of ETECST in LIF test (43.3) compared with control treatment (79.5) on mucosal (Duodenum) cellular immune response.

The assessment of mucosal LIF in migration percent to sensitizer was showing high significant LIF than systemic LIF, were the partially purified heat stable enterotoxin show the high level of Leukocyte migration inhibitory factor (54.5, 47.6 and 43.3) for systemic, appendix and duodenum consecutively, then crude enterotoxin with means (66.1, 52.6 and 54.6) for systemic, appendix and duodenum consecutively and the lower level of inhibition appear in killed antigen treatment (71.3, 57.5 and 56.1) for systemic, appendix and duodenum consecutively. All result were statistically significant at ($p<0.05$) compared with control (82.0, 79.5 and 79.5) in systemic, appendix and duodenum consecutively, tables (4), (5) and (6).

DISCUSSION

Erythrocyte-rosette Formation Test (E-rosette Test)

As known most T-lymphocytes have receptors for sheep erythrocytes (CD2) on their surface and can form 'rosettes' with them to identifying T-cells in mixed lymphocytes population. Leukocyte functional antigen-3 (LFA-3 now called CD58) is the surface molecule of sheep RBC that bind to (CD2) of T-lymphocytes [(12)]. The results showed that the formation of E-rosette in mucosal samples were higher than of systemic except for systemic killed antigen which induce E-rosette formation higher than of appendix and lower than of duodenum, while the ETECST showed the high level of E-rosette formation in appendix then in duodenum and in systemic. The crud enterotoxin showed low levels of E-rosette formation in systemic, appendix and duodenum consecutively. All results were statistically significant at ($p<0.05$) when compared with control tables (1), (2) and (3). These results consists with [(13)], who found that *Shigella dysenteriae* exotoxine was able to increase the number of T-lymphocyte due to formation of Erosette at

systemic and mucosal levels, and the mean values of mucosal were higher than that of systemic. Furthermore [8] reported that three different antigens of *E.coli* (outer membrane proteins, lipopolysaccharied and killed antigen) have the ability to significantly increase the levelof E-rosette formation compared with control. Effective immune defense requires CD4 T cells [14]. IFN-γ and TNF-α, which regulate T cell growth and differentiation and the recruitment and activation of inflammatory cells, respectively, which were involved in adaptive host defense against *C. rodentium* [15]. IL-12 and IFN-γ knockout mice significantly more susceptible to mucosal and gut-derived systemic *C. rodentium* infection. Analysis of the gut mucosa of IL-12 knockout mice revealed an influx of CD4-T cells and a local IFN-γ response. While IL-12 Critical cytokine for Th1 differentiation; induces proliferation and IFN-γ production by Th1 [16]. Heat stable enterotoxin may either form aggregates with other proteins or pigments or remain cell associated [17]. As illustrated in table (4) the crude enterotoxin showed very high level of lymphocyte activation (lymphocytosis) but not for E-rosette formation. However the CD2 marker may be blocked by heat-label particles preventing it from binding to its ligand on the sheep erythrocytes "leucocytes functional antigen- 3" (LFA-3) ,or adsorbed and bind sheep erythrocytes preventing them from binding to their receptors on T-cells.

Leukocyte Migration Inhibitory Factor (LIF) Test: Migration inhibitory factor (MIF) is considered to be the first lymphokine activity to be described. The simultaneous original descriptions by [18,19] were of a soluble factor purified from supernatants of activated lymphocytes that inhibited the random migration of guinea pig peritoneal macrophages. MIF was 'rediscovered' in the early 1990s as a cytokine/hormone released from the anterior pituitary in response to endotoxemia [20,21] found that MIF is stored preformed in significant quantities in human circulating granulocytes "eosinophils". In physiological stimulate, C5a and IL-5 cause significant release of MIF by eosinophils. The mucosal LIF was showing high significant levels than systemic, the ETECST showed the high level of Leukocyte migration inhibitory factor then crude enterotoxin and the lower level of inhibition appeared in killed antigen treatment. All result were statistically significant at ($p<0.05$) compared with control. Tables (4), (5) and (6). *E.coli* killed antigen showed significant level of LIF compared with control. Exotoxin of *Shigella dysenteriae* have the ability to induce specific cellular immunity at mucosal and systemic levels [13,8]. The significant decrease to more than 30% inhibition in index mean values of LIF at mucosal system may refer containing higher number of activated T-cell[22] Importantly, however, the clearance of intra tracheal administered Gram-negative pathogens was not only preserved, but slightly enhanced in MIF knockout mice. Plasma levels of the critical mediator, tumor necrosis factor α (TNFα), were lower in MIF knockout mice than in controls in response to septic insults [23]. The reduction of LIF with ETECST may be accordance with not full purification of toxin, where, Due to its small size, ST is nonimmunogenic in its natural form but becomes immunogenic when coupled to an appropriate large-molecular-weight carrier [24]. This has been successfully achieved with several carriers such as (B-subunit of LT, porcine IgG, B-sub unit of CT, ZZ moiety derived from *Staphylococcus aureus* protein A, Flagellin and porcine LT) were lost its toxicity and gain its specific immunogenicity [9].

CONCLUSIONS

Crud and partially purified enterotoxins have ability to induce cellular immune response in blood and mucosa.

RECOMMENDATION:

1-Prepare a carrier molecule model for partially purified enterotoxins

2-Make a vaccine against Enterotoxigenic *E.coli* toxins.

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