# Detection of specific antibodies of *Escherichia coli* O157:H7 isolated from children by using Eliza test

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**ABSTRACT**
This study comprises two parts: part one was conducted to isolate *E.coli* O157: H7 from children in Pediatric Hospital of Karballa city. The bacteria were identified by bacteriological, biochemical and serological methods.( 14 )isolates of *E.coli* O157:H7 isolated from children with diarrhea (230) and children had urinary tract infection(200) were isolated.
Part two of the study was included detection of antibodies against *E.coli* O157:H7 somatic (inactivated bacterium by heating) and flagellar (inactivated bacterium by formaldehyde) antigens in rabbits by using ELISA test. Twelve adult healthy rabbits were divided randomly for three equal groups A, B and C used for immunization study. All rabbits had negative fecal bacteriological culture of *E.coli* O157:H7 and serum was collected before immunization.
Serum antibodies titers were estimated by using ELISA and tube agglutination test pre and post each immunization. No antibody titers were detected Pre immunization in all rabbits. Elisa test revealed that the group (A) showed a significant variation between the value of antibody titers in the 2nd week and 4th week post immunization. Also in group (B) there was a significant variations between the antibody titers in the 2th week and 4th week post immunization. Group (A) revealed low significant ($p \leq 0.05$) antibody titers in serum (0.37±0.02) in comparison with group(B) which exhibited antibody titers in serum (0.44±0.04). No antibody titers was detected in rabbits of the control group (C) during the same weeks intervals. Antibody titers were estimated by tube agglutination test in immunized groups revealed significant ($p \leq 0.05$) antibody titers in serum (440±120) in group A in comparison with group-B which exhibited antibody titers in serum (560±80).Our study concluded the importance of detection Escherichia coli O157:H7 in children and that enzyme-linked immunosorbent posses the ability for detection antibodies against this bacteria.

# تقدير الأضداد الخاصة ببكتريا*E. coli* O157:H7 المعزولة من الأطفال باستخدام اختبار الاليزا

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**الخلاصة**
شملت هذه الدراسة جزئين: الجزء الأول أجري لعزل بكتريا الاشريشيا القولونية O157: H7 من الأطفال في مستشفي الأطفال في محافظه كربلاء. شخصت هذه البكتيريا بالطرق البكتريولوجية والكيميائية الحيوية والمصلية. تم عزل أربعة عشر عزله من اما الجزء الثاني من الدراسة فقد شمل الكشف عن الأجسام المضادة ضد مستضد *E.coli*157:H7 الجسمي المثبط بالحرارة وكذلك ضد المستضد السوطي المثبط بالفور مالين في الأرانب باستخدام اختبار تلازن الانبوب و اختبار الاليزا (ELISA). قسمت اثنا عشر أرانب كانت بالغه و بصحة جيده لثلاث مجموعات متساوية A، B و C استخدمت للدراسة المناعية. جميع الأرانب أعطت نتيجة سلبيه للزرع البكتريولجي وقد جمع المصل منها قبل التمنيع . حقنت المجموعة A بجرعة (1 مل) يحتوي على($10^8$ خليه /مل) من المستضد الجسمي تحت الجلد في الظهر بجرعتين خلال أسبوعين بين جرعه وأخرى, والمجموعة (B)جرعت بالمستضد السوطي (بنفس الطرق) ثم حقنت المجموعة الثالثة ( C) واحد مل من محلول دارئ الفوسفات الملحي المعقم كمجموعه سيطرة. تم تقييم مستوى الاجسام المضادة بوساطة اختبار التلازن الانبوب حيث اعطى زياده معنوية على مستوى ($p \leq 0.05$) واضحة للمجموعة الممنعة الثانية.(560±80) مقارنتا بالمجموعة الاولى (440±120)التي اعطت اقل مستوى بالأجسام المضادة في مصل الدم. وايضا تم تقدير مستوى الأجسام المضادة قبل وبعد التمنيع باستخدام فحص الأليزا. لم يظهر اي مستوى للأجسام المضادة قبل التمنيع في كل الأرانب, بعد التمنيع كشفت نتائج اختبار الاليزا أن المجموعة (A) أظهرت تباين كبير في مستوى الأجسام المضادة في الأسبوع الثاني والأسبوع الرابع بعد التميع ,وكذلك المجموعة (B )أظهرت تباين كبير في مستوى الأجسام المضادة في الأسبوع الثاني والأسبوع الرابع بعد التمنيع .المجموعة (A )أشارت إلى اختلاف قليل في مستوى الأجسام المضادة في المصل(0.37±0.02 ) بالمقارنة مع المجموعة B التي أظهرت مستوى الأجسام المضادة في المصل( 0.44±0.04) ولا يوجد مستوى للأجسام المضادة خلال نفس الفترة .

استنتجنا من هذه الدراسة أهميه الكشف عن الاشيريشيا المعوية *E.coli* O157:H7 في الأطفال وان فحص الاليزا يمتلك القدرة للكشف عن الأجسام المضادة ضد هذه البكتريا .

## 1- INTRODUCTION

Enterohaemorrhagic *E. coli* (EHEC) O157:H7 is a zoonotic pathogen of worldwide importance causing food born infections with possibly life-threatening consequences in humans, it is an emerging pathogen that causes acute human gastroenteritis and hemorrhagic colitis (1, 2, 3)

*Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli (*STEC) strains are important human pathogens that are mainly transmitted through the food chain. These pathogens have a low infectious dose and may cause life-threatening illnesses. However, detection of this microorganism in contaminated food or a patient's stool specimens presents a diagnostic challenge because of the low copy number in the sample (4).

Enzyme-linked immunosorbent assay (ELISA) was reported to quantitatively detect immunoglobulin G in 1971 according to(5) . Conventional ELISA (C-ELISA) has high reproducibility and possibility for the simultaneous quantification of a great number of assays, and is widely used to detect the presence of substances, including bacteria(6) .

ELISA is a rapid and reliable test which significantly reduces the time required to screen meats for the presence of *E. coli* 0157. Primary enrichment cultures grown for 8-16 hours can be tested in less than one hour, allowing ELISA-negative product to be released within 24 hours. Enrichment broths presumptively positive for *E. coli* 0157on the basis of positive ELISA tests can be cultured further for confirmation by standard methods(7) .

This study aimed to record the percentage of infection with *Escherichia coli* O157:H7 in children and to evaluate of Elisa test in detection of antibodies against somatic and flagellar antigens in rabbits.

## 2- MATERIALS AND METHODS.

### Samples collection .

Two hundred thirty fecal samples were collected from 230children suffering from diarrhea and bloody diarrhea and 200 urine samples from 200 children had a complication with urinary tract infection with both genders and different ages in Al-Hussain teaching hospital, in the province of Karbala,

### Bacterial examination

All samples were cultured on different selective media, then Gram stain was doing on the growing bacteria, confirmation by using biochemical test (KI, SC, Urease, Indole, Oxidase) and culturing on selective Chrom media specific for *E. coli* O157:H7 (8) .

### Latex agglutination Test for *E. coli* O157:H7:

**This test** was used for more specific identification of *E. coli* O157:H7 by using commercial kit (Wellcolex *E.coli* O157:H7, Remel) to detect the somatic antigen O157 and flagellar antigen H7 according to (9) .

### Preparation of somatic & flagellar *E. coli* O157:H7antigens:

These antigens were prepared according to (10): Initially, *E.coli* O157:H7 was cultured from the appropriate stock culture into nutrient broth and incubated at 37˚C for 24 hours, then the bacterial culture was tested to ensure their purity by preparing a slide of Gram stain and 0.5 ml from nutrient broth was transported to nutrient agar plate which spreaded by moving plates and left to dry then incubated at 37˚C for 24 hours. Bacteria were harvested with 5ml buffered physiological saline and transferred into sterile tube. The solution was centrifuged at 3000 rpm/15 minutes and washed with PBS for three times. Bacterial suspension turbidity was adjusted with 0.5 McFarland tube ($10^8$ cell/ml) and used for preparation of antigens as followed.

### Somatic Ag (Bacterial Inactivation by heating):

The prepared suspension of bacteria was put into the boiling water bath at 100˚C for 30 minutes. Then preserved at (-4˚C).

### Flagellar Ag (Bacterial Inactivation by formaldehyde):

Formaldehyde solution (0.6%) was added to the bacterial suspension and left overnight. Then washed three times with PBS to times to get rid of Formaldehyde. The antigen prepared was preserved at -4˚C.

**Sterility test:**

The prepared antigens were cultured on nutrient agar and examined regularly throughout the incubation period to test the sterility according to (11).

**Immunization of rabbits with antigens *E.coli* O157:H7.**

Twelve rabbits (local breed) at 6-9 months of age, weighting 1.5-3.0 Kg, were used in this study. They were obtained from the local market, housed in clean cages at room temperature; rabbits were adapted for two weeks before experiment start. At zero time prior to the inoculation Rabbits were examined clinically and serum samples were collected and kept in deep freez (-18C). All rabbits had negative faecal bacteriological culture for *E.coli* O157:H7. Then divided into three groups (4 rabbits for each group) Rabbits gave 2 booster doses at intervals 2 weeks ( Same rout and dose) :-

**A-First immunized group:**

Four rabbits were immunized subcutaneously with somatic antigen (O Ag) at a dose of 1ml containing $10^8$ c.f.u for two weeks intervals at two doses.

**B-Second immunized group:**

Four rabbits were immunized subcutaneously with flagellar antigen( H Ag) at a dose of 1ml containing $10^8$ c.f.u for two weeks intervals at two doses .

**C-Third group (control group):**

Four rabbits were injected subcutaneously with 1ml of phosphate buffer saline (PBS).

**Collection of blood samples for sera preparation .**

Five milliliter of blood were collected via cardiac puncture technique after cleaning and disinfection of the dragging sit from the two groups at zero, 14 and 28 days post infection according to method of(12). Each blood sample was put in gel tube and left until clotting, then kept in a refrigerator for overnight in standing position before serum collection by centrifugation (3000rpm) for 15 minutes and frozen at -18$^{\circ}$C till used . Estimation of Humeral immunity by ELISA test at zero, 2$^{nd}$ and 4$^{th}$ week of immunization.

## Tube agglutination test.

This test was done according to(13) .

Tube agglutination test consist of :

**1**-Rabbits antiserum against somatic antigen of *E. coli* O157and flagellar antigen of *E. coli* H7

**2**-O or H antigens from the test organisms were prepared at 6× $10^8$ / ml by a standard method.

**3-** normal saline**.**

**4-** *E.coli* strain was used as standard negative control.

## Procedures:

The tube agglutination test was done with normal saline in each 7 test tubes. Serial dilutions of Rabbits antiserum against somatic antigen of *E. coli* O157 in 0.5 ml volumes in normal saline were made then added O antigen from the test organisms was prepared at 6×$10^8$ / ml by a standard method. The final dilution from tube 1 to 6 is 1 : 20,1 : 40. . . 1 : 640; tube 7 was used as the control tube. All cultures which tested for the *E. coli* O157 antigen were also tested for the presence of the H7 flagellar antigen by the tube procedure. Multiple passages were done for flagellar enhancement and incubated at 37°C for overnight.

**ELISA test**

An ELISA was developed to monitor *E.coli* O157:H7- specific antibody responses in serum as follow:

**1**-Flat bottomed (96-well), microtiter plate was coated with 100 µl/well of *E.coli* o157:H7 strain ( 3x$10^8$ cells/ml, live cells) in 0.05 M carbonate buffer(pH 9.6) by incubating overnight at 4°C.

**2**-The plate was washed four times in phosphate- buffered saline containing 0.05% Tween 20 (PBST).

**3**-The bacterium was blocked with 150 µl/well of 2% bovine serum albumin in PBST for 1 hour at 37°C.

**4**-The plate was washed four times with PBST.

**5**- Serum (diluted 1:1000 ) in PBST was added 100µl/well to wells and the plate was incubated for 1.5 hour at 37°C.
**6**-The plates were washed four times in PBST .
**7**-One handred (100µl) of Peroxidase-conjugated sheep anti-rabbit immunoglobulin that diluted 1:3000 in PBS was added to each well. The plates were incubated for 1 hour at 37 °C.
**8**-Washed with PBST as before.
**9**-Then 100 µl/ well of freshly prepared substrate (0.06% o-phenylene- diamine) containing 0.21% hydrogen peroxide in a citrate phosphate buffer pH 5.0 was added. The reaction was allowed to develop for 10 min in the dark at room temperature and then stopped by the addition of 50 µl/well of 2 N sulphuric acid.
**10**-The absorbance was measured with a Titertek Multiskanreader at wavelength of 492 nm. (14)

The natural log of the Ab titer (Yi) in each serum were calculated as described by (15) .using the following linear regression equation:

**Yi** is the Ab titer of the each sample
**Pi** is the sample's optical density (OD) reading
**N** is the OD of the low (negative) standard in each plate

$$Yi = 0.32(Pi/N) - 0.32$$

## 3- RESULTS AND DISCUSSION

**Isolation of *E.coli* O157:**

Out of 230 stool samples and 200 urine samples examined, only 126 and 98 were positive for *E.coli* respectively from which 11(4.78%) and 3 (1.5%) isolates were *E.coli* O157:H7, respectively.

**Humoral immune response in rabbits.**

**Tube agglutination test:**

All groups had showed negative results for tube agglutination test before starting the experiment, but after immunization by (O Ag) and (H Ag) of *E.coli* O157 :H7, group (A) which was immunized by (O Ag) and group (B) which was immunized by (H Ag) showed different results of antibodies titers at 2nd& 4th weeks post immunization as shown in the(Table, 1,figure,1).

The rabbits which were immunized by (O Ag), after two weeks showed antibody titers with a mean of (220±60), but after four weeks, the antibody titers raised to reach a peak with a mean of (440±120).While the rabbits that were immunized by (H Ag) showed antibody titers after two weeks of immunization with a mean of (360±100.66) then after four weeks from immunization, the antibody titers raised to reach a peak with a mean (560±80) .No antibody titers in the rabbits of the control group (C) were observed during the same intervals.

Statistically, there was a significant inside and between groups at a level ($P \leq 0.0\ 5$). Antibody titers in immunized groups revealed lower significant ($p \leq 0.05$) antibody titers in serum (440±120) in group A in comparsion with group-B which exhibited antibody titers in serum (560±80).

**Table(1):** Means of antibody titers in the immunized and control groups.

| **Weeks after immunization** | **No.of the rabbits** | **Group (A)** | **Group (B)** | **Group (C)** |
|---|---|---|---|---|
| | | Immunization with **(O Ag)** | Immunization with **(H Ag)** | **PBS** |
| **0** | 4 | A 0 a | A 0 a | **A 0 a** |
| **2nd** | 4 | AB 220±60 a b | B 360±100.66 a | **A 0 b** |
| 4th | 4 | **AB 440±120 a** | **AB 560±80 a** | **A 0 b** |

The capital letters refers to the vertical statistical reading while small letters refers to horizontal reading.

**Figure (1):** Shows agglutination of (O&H) antigens by tube agglutination test.

a -Negative result of agglutination between (OAg) of *E.coli* and serum was prepared from immunized rabbits with somatic antigen of *E.coli* O157: H7.

b -Positive result of agglutination between (OAg) of *E.coli* O157: H7 and serum was prepared from immunized rabbits with somatic antigen.

c -Positive result of agglutination between (HAg) of *E. coli* O157: H7 and serum was prepared from immunized rabbits with flagellar antigen.

According to the above Strict evidence about tube agglutination test the results were compatible with Chapman, (1989) for detection antibody against O and H antigens of *E. coli* O157 H7.

**ELISA test**

Sera antibody titers which were detected by ELISA test in immunized and control groups at day 0, 2 weeks and 4 weeks showed in (Table,2; Figure,2 )

Prior to immunization, the three groups (A, B and C) of rabbits showed low peak of antibodies titers (0.05±0.01, 0.05±0.007and 0.05±0.006) respectively.

After two weeks of immunization with primary dose, group-A showed antibody titers with a mean of (0.19±0.008) and group B showed (0.28±0.01) and both immunized groups showed increased in antibody titer in comparison with the control group. After four weeks post immunization, the antibody titers were raised to reach a peak with a mean (0.37±0.02) in group A and group B (0.44±0.04). No antibody titers was detected in rabbits of the control group (C) during the same intervals.

Statistically, there was a significant ($P \leq 0.05$) variation inside and between groups . In the group (A) there was a significant variation between the value of antibody titers in the second weeks and fourth weeks post immunization. Also in group (B) there was a significant ($P \leq 0.05$) variations between the antibody titers in the 2$^{th}$ week and 4$^{th}$ week post immunization. Group A revealed low significant ($p \leq 0.05$) antibody titers in serum (0.37±0.02) in comparison with group-B which exhibited antibody titers in serum (0.44±0.04)

**Table (2):** Means of the antibody titers in rabbits immunized with O&H antigens of *E.coli* O157 H7.

| Weeks after immunization | No. of the rabbits | Group (A) | Group (B) | Group (C) |
|---|---|---|---|---|
| | | Immunized with O Ag | Immunized with H Ag | PBS |
| | | Mean ± SE* | Mean ± SE | **Mean ± SE** |
| **0** | 4 | A 0.05±0.01a | A 0.05±0.007 a | **A0.05±0.006 a** |
| **2 nd** | 4 | B 0.19±0.008a | B 0.28±0.01 b | **A0.04±0.004 c** |
| **4 nd** | **4** | **C 0.37±0.02a** | **C 0.44±0.04 a** | **A0.05±0.006 b** |

* SE: Standard error.

*The capital letters refers to the vertical statistical reading while small letters refers to horizontal reading.

**Figure (2):** Antibody titers of the three groups (A, B & C).

The present study revealed that 14 strains of *E.coli* O157 :H7 isolated from stool & urine samples were agreement with many researchers, *E.coli* O157:H7 was isolated by (16) with (5.7%) from stool of diarrheal patients in Basrah city, Other Iraqi studies such as (17) who found that *E.coli* O157:H7 serotyped as a cause of diarrhea in children with a ratio of 4% and study conducted by(18) who recorded that the proportion of *E.coli* O157:H7 isolation from diarrheal cases of children in USA was (3% ) but (19) reported the lower percentage of infection with *E.coli* O157:H7 in children (0.4 %). Also this result compatible with the result of (20) who showed that (2.3 %) of *E.coli* isolated from children's urine with UTI were

Enterohemorrhagic. A high rate reported by (21) who found (13%) among the children, received antibiotics and the hemolytic–uremic syndrome developed in (14%).

The present study has noticed that the experimentally immunized rabbits were able to induce humoral immune response which were represented by producing antibody against of *E.coli* O157:H7 and this production was elevated after two weeks from immunization and reached the peak after four weeks post immunization, and this was in agreement with (22) who found that the microbial antigens were able to stimulate the immune system,(23) found that the cellular and humoral aspects of immune response of the rabbits were stimulated by somatic polysaccharide and flagella of *E.coli* O157 :H7.(24) observed an impressive humoral immune response in immunized groups with *E.coli* O157H7 antigens of mice. Also( 25) showed the important role of antibody –producing B cell in protection against *E.coli* O157H7.(26) showed that flagellin was a strong T-cell activator so the flagellar antigen was more stimulated the immediate hyper sensitivity reaction compared with somatic antigens in immunized rabbits.

In this study immunization with O antigen evoked antibody response but less than that obtained from immunization with H antigen , this in compatible with several other studies that showed antibodies response against O antigen of *E.coli* O157 :H7 (24,27,28) suggested that the LPS O-antigen of EHEC O157:H7 played an important defensive role against antimicrobial factors in the host body fluid and was thus essential to the lethal effects of EHEC in animals.

The results of humoral immune responses to the prepared antigens in this study revealed that the antigens were affective in their action and gave sera antibody at different times of the study. Here, it was remarkably noted that enhancement in B-cell production was due to immune complex of antigens. The enhancement of this type of immune responses confirmed the potential of immune complexes to be used as vaccines.

**4-Conclusion**. Our study concluded the importance of detection *Escherichia coli* O157:H7 in children and that enzyme- linked immunosorbent posses the ability for detection antibodies against this bacteria.

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