Evaluation of home-mode ELISA system for the detection of antibodies against *Escherichia coli* O157:H7 using purified lipopolysaccharide

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Abstract

An enzyme linked immunosorbent assay (ELISA) for the detection and quantitation of human immunoglobulin G (IgG) antibodies against vero- cytotoxine (VT) producing Escherichia coli serogroup O157:H7 was produced. E. coli O157: H7 lipopolysaccharide was extracted from locally isolated strains by using hot phenol-water method, followed by partial purification using gel filtration chromatography by sepharose- 4B. The purity of the lipopolysaccharide was checked by measuring the protein and nucleic acid content and then used as antigen. Four isolates of vero-cytotoxin producing E. coli serogroup O157:H7 was obtained by culturing 350 stool samples from children suffering from bloody diarrhea. These isolates were identified on bacteriological, serological and biochemical basis. Toxin production was confirmed on laboratory animals as well as by cytopathic effect on tissue culture. The possibility of using E. coli O157:H7 lipopolysaccharide in an enzyme- linked immunosorbent assay for the routine diagnostic testing of serum from patients for evidence of O157:H7 infection is discussed.

Introduction

The bacteria E. coli O157 was first identified as a human pathogen in 1982 [(1)] which is responsible for the majority of cases of enterohaemorrhagic disease [(2)]. It is transmitted to human through contaminated food, water, and direct contact with infected people or animals [(3)]. Effective prevention of the disease is crucially dependent on rapid detection of the causative pathogen. Due to various strain differences and heterogeneity reliable identification of Enterohemorragic E.coli EHEC by culture methods is almost impossible [(4)]. Another problem for diagnostic laboratories is that EHEC has an extraordinary low infectious dose. That is why many techniques have been developed for diagnosis of infection with these bacteria including serology, immunomagnetic separation, and PCR[(4)]. The present paper describes the preparation of home-made ELISA system for the detection of antibodies Ab against E. coli O157:H7.

Materials and Methods

• Patients and bacterial isolates

Through a period extended from the 1st of June 2001 till the end of September of the same year, stool from 350 children suffering from diarrhea, who were referred to AL- Alwiya children hospital, were cultured looking for possible bacterial causes. Only stool samples containing pus and red blood cells were included. Serum samples were collected from those patients and kept frozen at -20°c till required. Ninety apparently healthy children were included in the study as control group.

Tests performed were general stool examination and cultures for the identification of *Salmonella* and *Shigella*, *Campylobacter spp*. and for *E. coli* O157:H7.

Culture for EHEC was done on sorbitol MacConkey as described by Johnson,*et.al* [5].suspected colonies were confirmed by biochemical reactions including cellobiose test, KCN test, Enterohemolysin test and MUG test (4- Methylumbilifery B- D-glucuronide). Further serological confirmation was done using rapid agglutination test (Well Colex).

Toxin purification was done as described by [6]. Briefly the isolates were inoculated in Tryptone Soya broth, incubated at 37ºc for 24 hours in a shaking water bath. The supernatant was collected by centrifugation at 10000 rPM for 30 minutes, and sterilized by filtration.

Toxin effect was studied on suckling albino mice and cytopathic effect on vero cell line as described in a previous study [7].

- **Lipopolysaccharde extraction and purification:**

Lipopolysaccharide from EHEC was extracted as described by Galanos,et.al, [8]. Briefly; the bacteria were inoculated in nutrient broth for 24 hours at 37ºc. The resulting growths harvested by centrifugation and washed once with distilled water,alcohol, and acetone and twice with either and then dry with routary evaporator at 30-40ºc. The LPS extraction with hot phenol- water was carried out by the method of extraction from the water phase was used.

Partial purification was done using gel filtration chromatography by sepharose 4-B [9].

The purity of the partial purification lipopolysaccharide was checked by measuring the protein content as described by Bradford,*et.al*, [10] with bovine serum albumin as the standard and by measuring contamination of nucleic acid spectrophotometrically (260 nm).

- **ELISA technique:**

ELISA procedure was done according Jenkins,*et.al*, [11]. EHEC LPS, diluted at different concentrations with cating buffer, was adsorbed on polystyrene Microplate. The plates wer incubated at 4ºc over night. Blocking was done next day using phosphate buffered solution containing 0.05% Tween 20 and 5% bovine serum albumin. Diluted serum samples were added after blocking and the plates were incubated for one hour at 37ºc. One hundred µl of the conjugate (horseradish peroxidase) was added after washing and the plate was further incubated for one hour at 37ºc. A TMB substrate was added to the plates after washing and incubated at a dark plan for 30 minutes when a 50µl of stopping solution was added before reading at 450 nm. Chequer-board titration was done to define the optimum concentration of the LPS and the optimum serum dilution. Cut- off value was calculated by adding three standard deviation of the optical density to the mean of the reading of serum samples.

RESULTS

- **Evaluation of purified LPS:**

Out of the 350 stool samples cultured, only four cases were diagnosed as being infected with EHEC. These diagnoses were confirmed by the above mentioned biochemical and serological tests. Toxin production was confirmed by its effect on suckling mice and by its cytopathic effect on vero cell line.

Partial purification for the LPS was done by gel filtration chromatography. Two peaks were evident, one at the parts 14-17 and the other at the parts 57-60. Only one carbohydrate peak

was seen at the parts 14-30 by measuring carbohydrate concentration in separate parts of the column [12] as shown in figer1 According to these results the parts 18-28 represents the partially purified LPS.

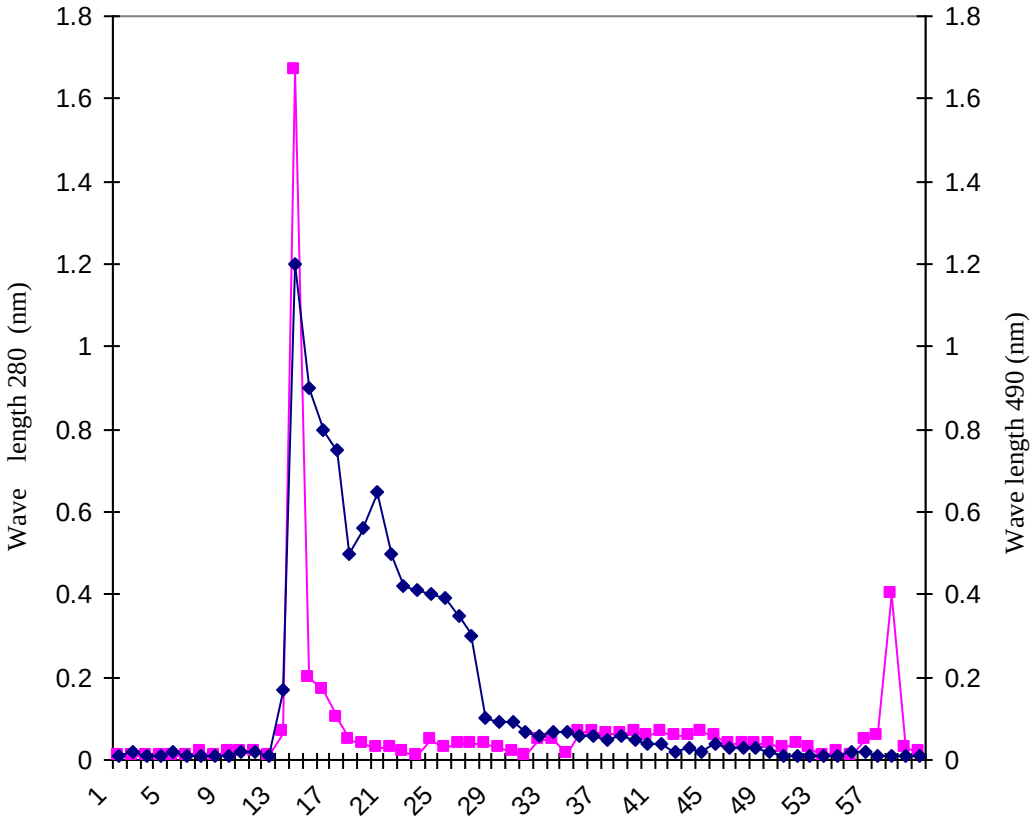

Figure (1) :Gel electrophoresis chromotographi for extracted LPS from local isolates of E.coli O157:H7 using Sepharose – 4B

Carbohydrate ♦ , Protein ■

As for the ELISA system, chequer board titration showed that the optimum LPS concentration used as antigen was 0.1µg/ well and that the optimum serum dilution that gave best discrimination between positive and negative control was 1:100. Concentration calculation of the cut-off value, the mean OD of the control group (90 child) was 0.275 with standard deviation of 0.083. Thus the calculated cut-off value was 0.524 (mean + 3Sd). Using this cut-off value, four cases turned out to be positive when the test was applied to the 350 children with diarrhea figer2 shows the distribution of the optical density of the three groups involved in the study, the first is the control group and the second is the 6 children who were positive for the ELISA test (mean OD of 0.665 with a SD of 0.06). The last group includes the 344 children with diarrhea who were negative by the ELISA test (mean Od of 0.300 with a Sd of 0.088). Statistical analysis showed significant differences between these groups (P< 0.001).

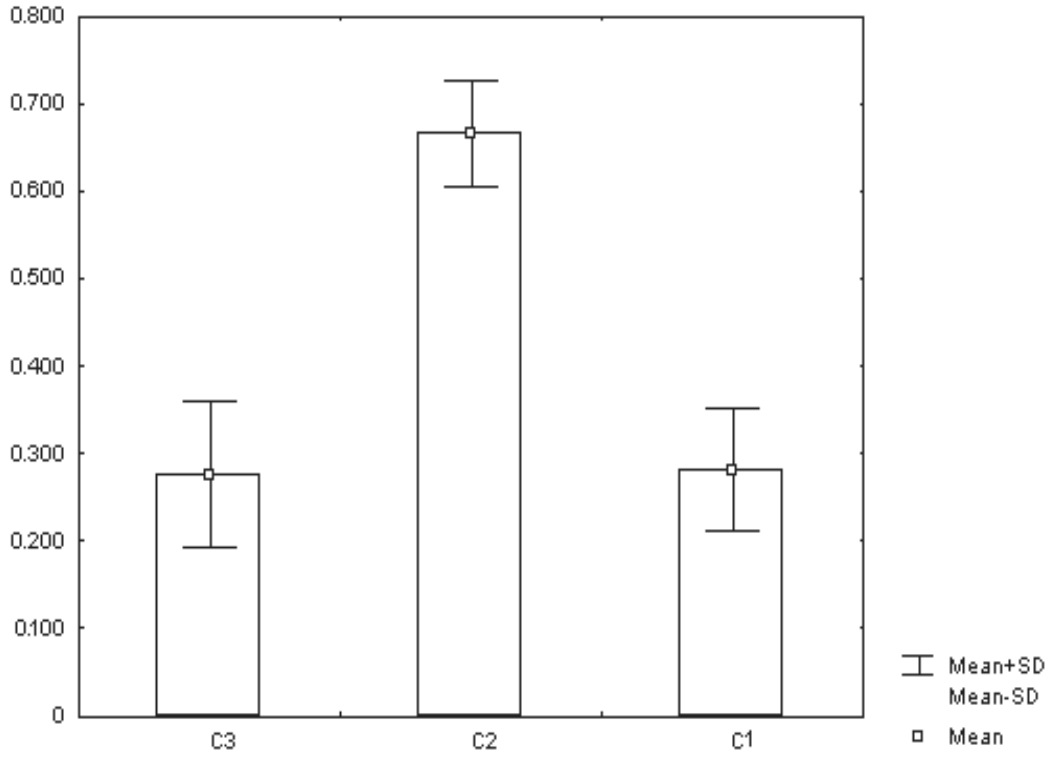

Figure (2):Distribution of optical density for 350 childern
C1 = No.of patient children seeking the hospital during the study for anather diseases (control group)
C2 = No.of patient children (positive group)
C3 = No.of patient children (Negative group)

Discution

It is worthly to mention that this study clearly demonstrated the possible application of home- made ELISA in the diagnosis of EHEC infection. This system was capable of detecting all the cases [(4)] of EHEC infection proved by culture. The other two cases that were positive by ELISA could be a true infection with these bacteria that have missed by culture technique. This is possibly due to the low infection dose with these bacteria. On the other hand this result could be due to possible cross reaction with other E. coli infection of possibly related Enterobacteriaceae.

Of importance to notice is the small number of cases of EHEC infection proved by culture or serologically in this study could be due to the following points:

1. Possible misuse of antibiotics, which have been given to the patients prior to taking sample for culture, a trend that is common in this country.
2. One of the known facts about these bacteria are the differences in the geographical distribution of infection. There is a reported difference in the incidence of cases of EHEC, with reduced number of reported cases as we move from the Western and Northern parts of Europe, passing down to the Mediterranean area(3).
3. Similarly, number of reported cases varies with the season of the study, and an increased incidence of cases is reported in the summer months of the year.

Our study concolution a reduced in incidence of cases of diarrhea due to EHEC. Serological diagnosis could be of help to assist the diagnosis of these infections. For proper assessment of the incidence of this bacteria, introduction of detecting these bacteria as a part of the routine culturing system in dealing with bacterial causes of diarrhea in pediatric age group.

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تقييم عدة تشخيصية لعزل وتشخيص بكتريا *Escerichia coli O157:H7*

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الخلاصة:

خلصت هذه الدراسة الى امكانية استخدام عديد السكريد الشحمي المستخلص من بكتريا *E. coli*: O157:H7 في تقنية الاليزا كاختبار تشخيصي وروتيني للمصول المأخوذة من المصابين كدليل على الاصابة بهذه البكتريا.

استخدمت تقنية الاليزا للتشخيص والكشف عن الاضداد (الكلوبيولين المناعي نوع IgG) المتولدة في جسم الانسان ضد الذيفان الخلوي Verocytotoxin المنتج من قبل بكتريا *E. coli*: O157:H7 .

استخلص عديد السكريد الشحمي (Lipopolysaccharide) من السلالات المعزولة محليا عن طريق استخدام طريقة الماء –الفينول الساخن والتب تبعتها تنقية جزيئة باستخدام جهاز الفصل gel filtration chromatography وباستخدام Sepharose-4B وتم تشخيص وتأكيد نقاوة Lipopolysaccharide عن طريق قياس محتوى البروتين والاحماض النووية والتي استخدمت فيما بعد كمستضد (Antigen).

تم الحصول على اربعة عزلات من بكتريا *E. coli*: O157:H7 والمنتجة للذيفان الخلوي Verocytotoxin عن طريق زرع 350 عينة براز من اطفال عانوا من الاسهال الدموي وهذه العزلات تم تشخيصها بالاعتماد على الاسس البكتريولوجية والسيرولوجية والكيموحيوية. تم تأكيد الدلالة على انتاج هذا الذيفان على الحيوانات المختبرية فضلا عن التأثيرات الخلوية في المزارع النسيجية.