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Evaluation of Some *Escherichia Coli* Antigenic Components as A Discriminatory and Diagnostic Tool

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ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of diarrhea in newly born animals in developing countries and could be transmitted to children and stressed adults as travelers through contaminated meat and milk. Continuous trials were performed to prevent this disease using different antigenic determinants. This study was conducted at three *E. coli* serotypes O78, O125, and O158, which were isolated from diarrheic calves, buffalo calves and lambs in Egyptian farms, to extract periplasmic protein and lipopolysaccharide and assessed their antigenic and diagnostic performance. SDS-PAGE electrophoresis was conducted on crude extracted periplasmic proteins from the isolated serotypes O78, O125, and O158, and revealed 8,9, and 9 bands respectively, ranged from 43 to 257 kDa with sharing in 5 bands and the molecular profile nearly similar between the three serotypes. Native SDS-PAGE silver stained electrophoresis was conducted on extracted crude lipolpolysaccharides and showed 10,7, and 3 bands for serotypes O78, O125, and O158 respectively ranged from 25 to 168 kDa with sharing in 3 bands. Agar gel immunodiffusion test was carried out on the extracted crude periplasmic proteins and lipopolysaccharides from the three *E.coli* serotypes, against *E.coli* antisera previously prepared in rabbit, and the studied both antigens gave precipitin lines suggesting that the shared fractions appeared in electrophoresis may responsible for the production of antibodies and may be considered a tool of diagnosis and future vaccine.

KEY WORDS: *Escherichia coli*, periplasmic, lipopolysaccharide, electrophoresis, Agar agel immunodiffusion.

1. INTRODUCTION

Acute diarrheal disease is an issue that impacts adults and children as well as newly born animals and *Escherichia coli* is among the remarkable bacterial causes of dehydrating diarrhea (Perez, 2010). There are six pathotypes of *E. coli* have been embraced in diarrheal processes, the enteropathogenic (EPEC), and enterotoxigenic (ETEC) strains are the most concern due to their zenotic tendency as the diseased farm animals can play a role in transmission through contaminated milk and meat (Farfan-García, 2016). In developing countries, increasing the age is accompanied with the decreasing in *E. coli* clinical infection incidence, suggesting that immunity to pathogenic *E. coli* infection can be acquired and proposing that an access to vaccination may steady successful (Kumar, 2015). Bacterial surface proteins are fascinating as vaccine components objective, however, identifying these proteins can be hard, and need intricate separation procedures (Darsley, 2012).

The periplasm of Gram-negative bacteria as *E. coli* comprised a number of specific binding proteins that play not only physiological roles such as helping the organism to survive for a time in the low pH conditions of the mammalian stomach, copper and silver resistance, envelope stress responses, but also are considered an important virulence factor; provide resistance to a variety of antimicrobial agents and have a chemotactic effect (Elkins, 2003; Hong, 2005; Loftin, 2005). Another important outer membrane component is the lipopolysaccharide (LPS) which is considered a potent immunogenic endotoxin result in induction of interferon and tumor necrosis factor and pathogen-associated molecule. (Cristiano, 2013) In the current study, the are trials to extracte the lipopolysaccharide and periplasmic protein from local *E. coli* serotypes and assess their diagnostic and protective features.

2. METHODOLOGY

Sample: three different *E. coli* serotypes O78,O125, and O158 that isolated from diarrheic newly born calves, buffalo calves, and lambs in Egyptian farms.

Preparation of *Escherichia coli* Antisera in Rabbits: The anti sera were prepared in 2-2.5 kg New Zealand white rabbits by intravenous injection of acetone killed unknown *E.coli* isolate at days; 1, 5, 10, 15, 20 by a dose of 0.5ml, 1ml, 2ml, 2ml, 2ml respectively. Rabbits were bled from the ear vein at days 25, 30 and bled out on day 35 and the antibody fractions were precipitated by bringing it to 50% saturation with ammonium sulfate (Brade, 1986).

Extraction of Crude Periplasmic Protein From Subtyped *E.Coli* Strains: The serotyped *E.coli* isolates were cultured onto the nutrient agar plats for 24 hours at 37ºC. The grown colonies were harvested, washed three times using 0.15M sodium chloride, then centrifuged at 3000 rpm for15min. Each pellet was resuspended using the same previous buffer, then autoclaved at 121ºC for 20 minutes. The suspension of each autoclaved serotype was centrifuged at 12000 rpm for 30 minutes, and then each supernatant was separated. Ammonium sulfate was added to each supernatant to obtain a final 100% saturation. The precipitated proteins were collected by centrifugation at 3000 rpm for15 minutes, then the pellets were resuspended in 0.01M phosphate buffer saline (PBS) and dialysis occurred

against PBS in a dialysis bag for 2-4 days at 4ºC. Each solution was aspirated, protein concentration determined and stored at -70ºC till used (Fulda, 1999).

Extraction Of Crude Lipopolysaccharide Antigens: The serotyped *E. coli* isolates were cultured onto the nutrient agar plates for 24 hrs at 37ºC.The grown colonies were harvested in 20ml sterile distilled water for each and autoclaved at 121ºC for 20 minutes. Each suspension was centrifuged; at 12000 rpm for 45 minutes.The supernatants were collected and filtered through 0.45 Millipore filter. Four molar solution of sodium hydroxide was added to each supernatant about 1:15 volume to volume and heated at 56ºC for 1 hour, then the solutions were cooled at room temperature and neutralized to pH 7 by using glacial acetic acid. Ethyl alcohol 70% was added to each solution in a ratio 4:1 and standing at 4ºC for 24-48 hours.The precipitates were collected, centrifuged at 3000 rpm for 5 minutes, then dissolved in small amounts of sterile distilled water and dialyzed for 48 hours at 4ºC against distilled water, after dialysis the solutions were stored frozen at -20ºC till used (Berman, 1980).

SDS-PAGE Electrophoresis: The prepared periplasmic proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% NuPage Bis-Tris gradient gels (BIO-RAD). The gel was removed from 5% glycerol solution and drying occurred in a sequenced manner (porous support, dry filter paper, transparent gasket then a assembling the drying condition 25in-Hg, 80ºC, 2 hours.

Silver Stain for Prepared Lipopolysaccharide: This was carried out on Native-Page (without SDS and 2- mercaptoethanol)**,** using the special stacking and resolving gels previously described and electrophoresis was done at 60 mA. The run was continued till the dye come to the bottom of the plate, and the gel was transferred to fixing solution overnight. The gel was gently rocked for 5 minutes in freshly made 0.7% periodic acid dissolved in fixing solution. Then the gel was rinsed two times in deionized water for 6 minutes and silver stained (Tsai and Frasch 1982). Quantitization of different fractions of each sample were performed using BIO-RAD (GS- 700 Imaging Densitmeter) by molecular analysis software.

Agar Gel Immunodiffusion Test:

Optimization of the antigens concentration: One gram of purified agarose was dissolved in 100ml of borate sodium chloride buffer pH 8.3 containing 1% sodium azide, heat dissolved and the solution poured in clean and dry Petri dishes in 0.4mm thickness then lifted to solidify. Standard wells 0.6mm diameter and 0.4mm apart was punctured in the agar using a standard Rosetta. The prepared *E.coli* antiserum was added in the central well and different antigen concentrations were added in the surrounding wells, then incubated at 37ºC for 24-48 hours in humid incubator. The clear precipitation line or lines facing the antigen dilution was considered the best dilution used. The extracted crude periplasmic protein and lipopolysaccharide antigens of untyped *E.coli* in optimum concentration were added in the central well and the prepared *E.coli* antiserum was added in the surrounding wells then incubated at 37ºC for 24-48 hours in humid incubator. After the lines appeared, the dishes were preserved at 4°C and reading occurs through the uniform light source (Alton, 1988).

3. RESULTS

SDS-PAGE Electrophoretic Analysis of Crude Periplasmic Proteins Extracted from The *E.Coli* Serotypes Isolates: Crude periplasmic proteins extracted from isolated serotypes O78, O125, and O158 were analyzed with SDS-PAGE electrophoresis.The results are shown in Fig.1. The approximate molecular weight of the protein fractions as compared to those of the standard marker is illustrated in table.1, from which, one can conclude that periplasmic proteins extracted from serotype O78 revealed 8 bands with a molecular weight ranging from 43 to 235 kDa. On the other hand, *E.coli* serotype O125 showed 9 bands with a molecular weight ranging from 50 to 257 kDa, while *E.coli* serotype O158 displayed 9 bands also, but with a molecular weight ranging from 50 to 249 kDa. The obtained results demonstrated that there are 5 common shared bands and the molecular profile nearly similar between the three serotypes.

Table.1. Electrophoretic pattern of crude periplasmic proteins extracted from *E.coli* serotypes O78, O125, and O158

Bands	Serotypes			Bands	Serotypes		
	O78 M.w.	O125 M.w.	O158 M.w.		O78 M.w.	O125 M.w.	O158 M.w.
1	235	257	249	7	76	76	76
2	222	222	222	8	70		
3		166	166	9		55	55
4	137	151	143	10	50	50	50
5		105	110	11	43		
6	90	80	85				

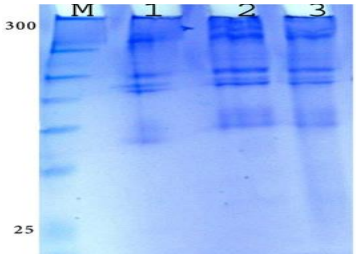

Figure.1. Electrophoretic pattern of extracted crude periplasmic proteins: lane 1: marker. lanes 2, 3, and 4 represented serotypes O78, O125, and O158 respectively.

Native SDS-PAGE Electrophoretic Analysis of Crude Lipopolysaccharides Extracted from Isolated 3 Serotypes of *E.Coli*: Extracted crude lipopolysaccharides from serotypes O78, O125, and O158 isolates were analyzed with native SDS-PAGE electrophoresis using silver stain. The results are shown in Fig.2, the approximate molecular weight of the fractions as compared to those of the standard marker is illustrated in table (2). The obtained results showed that lipopolysaccharides extracted from serotype O78 revealed, 10 bands with a molecular weight ranging from 25 to 167 kDa. On the other hand, *E.coli* serotype O125 showed 7 bands with a molecular weight ranging from 29 to 168 kDa, while *E.coli* serotype O158 displayed 3 bands with a molecular weight ranging from 29 to 33 kDa.The results demonstrated that there are 3 common shared bands among the three serotypes.

Table.2. Electrophoresis pattern of crude lipopolysaccharides extracted from *E.coli* serotypes O78, O125, and O158

Bands	Serotypes		
	O78 M.w.	O125 M.w.	O158 M.w.
1	167	167	
2	156	160	
3	150		
4	136		
5	109		
6		70	
7	52	50	
8	35	35	35
9	32	32	32
10	29	29	29
11	25		

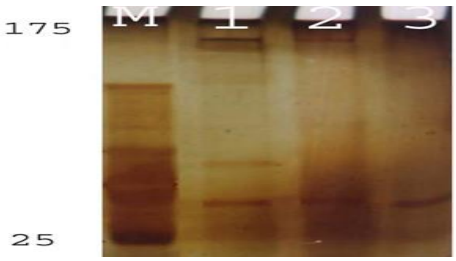

Figure.2. Electrophoretic pattern of extracted crude lipopolysaccharides: lane 1: marker. lanes 2, 3, and 4 represented serotypes O78, O125, and O158 respectively.

Agar Gel Immunodiffusion Test Using Different Extracted *E.Coli* Crude Periplasdmic Proteins: Agar gel immunodiffusion test was assayed on the extracted crude periplasmic proteins from the three *E.coli* serotypes, O78, O125, and O158 using *E.coli* antisera previously prepared in rabbit. Fig.3, showed that there were lines of positive results between the antigen and antisera wells.

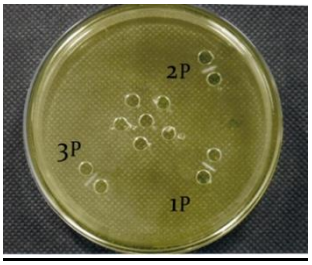

Figure.3. Agar gel immunodiffusion test using different extracted *E.coli* crude periplasdmic proteins showed formation of the precipitation lines

Agar Gel Immunodiffusion Test Using Different Extracted *E.Coli* Crude Lipopoysaccharides: Agar gel immunodiffusion test was done on the extracted crude lipopolysaccharides from the three *E.coli* serotypes, O78, O125, and O158 using *E.coli* antisera previously prepared in rabbit. Figure.4, showed that there were lines of positive result between the antigen and antisera wells.

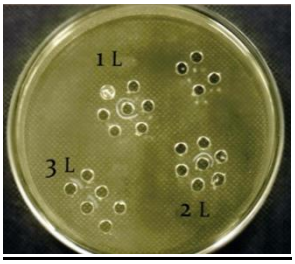

Figure.4. Agar gel immunodiffusion test using different extracted *E.coli* crude lipopolysaccharide showed formation of the precipitation lines

DISCUSSION

Enterodiarrheic *E. coli* are involved in diarrhea in newly born animals, as well as infants, one of the virulence factors of these bacteria is the secretion of certain periplasmic protein taken part in microvilli adhesion and effacement (Shimizu, 2015).

Periplasmic proteins are groups of surface proteins found in periplasmic space involved in various pathogenesis processes, as biofilm formation, capsular biosynthesis which responsible for resistance of phagocytosis, also has a chemotactic effect (Collins, 2006; Dorel, 2006). The data in table.1 and figure.1, shows the electrophoretic pattern of the extracted periplasmic protein of the three *E.coli* serotypes O78, O125, and O158 isolated from diarrheic newly born calves, buffalo calves, and lambs. The data demonstrated 8, 9, and 9 bands for serotypes O78, O125, and O158 respectively ranging from 43.6 to 257.2 kDa, with 4 common shared bands which are 48.845-50.789, 75.66-77.67, 82.8-86.19 and 220.8-222.6 kDa. These findings nearly coincide with the investigations of (Khodabakhsh, 2013).

Lipopolysaccaride (LPS) is the main component of the outer membrane of the cell envelope of Gram-negative bacteria. LPS has an endotoxic effect result in the inception of different activities as induction of interferon and tumor necrosis factor, so it is considered an immune-stimulant agent, represented O-antigen therefore be a target for new diagnostic and preventive strategies (Gamage, 2004**).** LPS is usually extracted in previous investigations using hot water-phenol method, but in this study the extraction would be done successfully by the distilled water method. The results are shown in figure.2 and table.2. The obtained data revealed10, 7, and 3 for serotypes O78, O125, and O158 respectively ranging from 25.65 to168 . 25 kDa. The results demonstrated that there are 3 common shared bands among the three serotypes. These data tend to agree with those of (Datta, 1999; Wang, 2010).

Agar Gel Immune Diffusion test (AGID) is a very simple in comparison to other serological tests which can be carried out with little amount of serum besides that contaminated or hemolytic samples which are unsuitable for ELISA and SAT could be used in AGID. Moreover, it does not give any non specific reaction that often observed with SAT but gives a precipitin line in only high titer, not in doubtful or low titer cases. In the present study, the AGID was done on the three extracted periplasmic proteins of the *E.coli* serotypes O78, O125, and O158 with *E.coli* antisera prepared in rabbit. The results in figure.3, revealed that the studied periplasmic proteins gave precipitin lines against antisera suggesting that the shared fractions appeared in electrophoresis may responsible for the production of antibodies and this agree with large extent to the investigations of (Khodabakhsh, 2013; Sprencel, 2000; Alipour, 2009; Noor, 2009) which their findings indicated that the recognized periplasmic proteins are potentially useful for serodiagnosis and may serve as targets for vaccine design ranging from 38kDa to 52kDa.

Furthermore, the results in figure.4, revealed that the three extracted lipopolysaccharides of the *E.coli* serotypes O78, O125, and O158 gave precipitin lines against *E.coli* antisera prepared in rabbit, implying the same suggestion that the shared fractions appeared in electrophoresis may responsible for the production of antibodies, the results go hand to hand with the data (Jolanta, 2002; Chart, 2004; Müller-Loennies, 2004; Denoncin, 2014).

4. CONCLUSION

Finally, the present study showed extraction of some antigenic structure of *E.coli* as periplasmic protein and lipopolysaccharides and seems to be useful in serodiagnosis of different O-strains, and it is recommended to perform further investigations to achieve more purification and fractionation of these antigens to fullfil a proper future vaccination.

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