

Hyperimmune lipopolysaccharide antiserum mediated inhibition of the adherence of *E. coli* O157:H7 to HEP-2 cells and large intestine of mice

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Summary

Escherichia coli O157:H7 is found in cattle farms and can live in the intestine of healthy cattle. Most cases of human illnesses including nonbloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome can be traced, either directly or indirectly, to cattle. One strategy for reducing the risk of *Enterohemorrhagic Escherichia coli* (EHEC) infections in human is to reduce the prevalence of infection in cattle. Antiserum against whole cell of isolated *E. coli* O157:H7 from cattle showed inhibition of adherence of this strain to HEP-2 cells and intestine of model mice. The ability of this antiserum in blocking the attachment of bacteria to HEP-2 cells in 1:1280 titer and to intestine tissue of mice in 1:640 titer are significant. Histology of intestine tissue confirms our results. The difference between *in vivo* and *in vitro* titrations for blocking the attachment depends on these two different conditions.

Key words: *Escherichia coli* O157:H7, Hyperimmune lipopolysaccharide antiserum, Inhibition, Adherence, HEP-2 cells

Introduction

Enterohemorrhagic Escherichia coli (EHEC) serotype O157:H7 were first associated with human disease in two community outbreaks of hemorrhagic colitis (Riley *et al.*, 1983). Also, this serotype has been associated with a spectrum of sporadic and endemic human illnesses ranging from nonbloody diarrhea to the hemolytic uremic syndrome. Cattle are important reservoirs of EHEC O157:H7 strains (Su and Brandt, 1995). The majority cases of EHEC disease are associated with ingestion of undercooked, contaminated hamburger or raw milk. One strategy for reducing the risk of EHEC infections in humans is to reduce the prevalence of infection in cattle. Epithelial cell cultures serve as models to evaluate the mechanisms by which enteric pathogens interact with eukaryotic host cells *in vivo*. One of the epithelial cell lines that has been

used to analyze the adherence of pathogenic *E. coli* is HEP-2 (human larynx) cells (McKee and O'Brien, 1995). *Escherichia coli* O157:H7 also demonstrate intimate adherence to enterocytes in animal models of human disease (Sherman *et al.*, 1988). At sites of bacterial adherence to the apical plasma membrane, there is actin accumulation and disruption of normal microvillus membrane (Knutton *et al.*, 1989). *Escherichia coli* O157:H7 can adhere to epithelial cell lines in tissue culture. This strain does not demonstrate either invasive properties or localized internalization. The objective of the present study was to determine if this strain, isolated from cattle, produces changes in HEP-2 cells and the intestine of the model mouse, and if the hyperimmune antiserum against lipopolysaccharide (LPS) blocks attachment of bacteria to these cells.

Materials and Methods

Bacterial strains

Escherichia coli O157:H7 was isolated from cattle. Stock culture of the strain was subcultured in Luria broth and incubated aerobically for 18 h at 37°C. The ability of the bacteria to produce stx1 and stx2 was examined using a commercial kit (VTEC-RPLA, Oxoid Inc.) in accordance with the manufacturer's instructions.

HEP-2 cell culture

Human laryngeal epithelial (HEP-2) cells were maintained by serial passage in Eagle's minimal essential medium (EMEM), supplemented with 10% fetal calf serum, 20 μ l-glutamine, 100 μ g/ml gentamicin, 10 units/ml penicillin G and 10 μ g/ml streptomycin (called complete EMEM). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂. HEP-2 cells were seeded into 8-well chamber slides at a density of 3×10^4 cells/well and incubated for 72 h. The medium in each well was then removed and replaced with complete EMEM that contained 1% fetal calf serum. Cells were maintained under these conditions for up to 48 h prior to use (Knutton *et al.*, 1997).

Antisera

Hyperimmune LPS antiserum was prepared by injecting an adult female rabbit from the Razi Vaccine and Serum Research Institute (RVSRI) colony. The rabbit was injected intraperitoneally with heat-killed *E. coli* O157:H7 emulsified in complete Freund's adjuvant. Two weeks later, the rabbit was reinjected in the same way with incomplete Freund's adjuvant. A third injection was done in the same way two weeks later. The last injection was done in vein without adjuvant and the rabbit was exsanguinated two weeks later. Immunized serum was pooled, sterile filtered, and stored at -20°C.

The experiments comply with the current laws of the country in which they were performed.

Adherence assay to HEP-2

Infection of HEP-2 cells with EHEC strain O157:H7 was done with minor

modification. Briefly, HEP-2 cells monolayers were infected with this strain taken from a Luria broth overnight culture and diluted into EMEM containing 1% mannose and 10 mM sodium phosphate without 0.4% sodium bicarbonate (Cravioto *et al.*, 1979). After 2.5-3 h of infection, cells were washed with phosphate-buffered saline (PBS) to remove nonadherent bacteria and fresh EMEM buffer was added. After an additional 3 h of incubation, the infected cells were washed thoroughly with PBS to remove all bacteria that were not intimately adhered. Cells were then fixed and prepared for hematoxylin and eosin (H&E) staining and scanning electron microscopy (SEM). To assess the effect of antiserum on *E. coli* O157:H7 adherence, hyper immune LPS antiserum was added to the bacteria suspended in 1 ml of adherence medium and the bacterium-antiserum mixture was incubated at 37°C for 30 min prior to infection of the HEP-2 cells.

Scanning electron microscopy (SEM)

Following the adherence assay, samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, post-fixed in 1% aqueous osmium tetroxide and dehydrate in 2, 2-dimethoxy propane. Specimens were transferred to absolute ethanol, critical-point-dried using liquid carbon dioxide and were examined under a DSM 940A, ZEISS scanning electron microscope (Phillips *et al.*, 2000).

Transmission electron microscopy (TEM)

Large intestine were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% aqueous osmium tetroxide, and embedded in TAAB resin. Thin sections were double stained with uranyl acetate and lead citrate and viewed under a DSM940A ZEISS transmission electron microscope at 50 KV (Phillips *et al.*, 2000).

Challenge of mice with bacteria and bacteria-antiserum

Ten 21-day-old female Razi mice were orally challenged with a gavage needle with *E. coli* O157:H7 isolate, grown with shaking at 37°C. For inoculation, bacteria were grown overnight in L broth containing 100

$\mu\text{g/ml}$ of nalidixic acid. The bacteria were diluted with phosphate-buffered saline (pH = 7.2) to OD₆₀₀ of 1.7 and delivered to mice in a volume of 100 μl (Ca. 1.5×10^7). At day 15 of postchallenge, the surviving mice were used to determine the level of colonic colonization and the degree of histopathological changes. An additional 10 mice were infected with *E. coli* O157:H7 and antiserum. For this, bacteria and antiserum were mixed and incubated at 37°C for 30 min before challenge. Tests for colonic colonization were done day 15 post challenge. Ten mice were also challenged with PBS as the control group.

Histology

The 10 mice picked from the group infected with *E. coli* O157:H7 were sacrificed day 15 postchallenge. The large intestine was removed, opened longitudinally, and rinsed in PBS. A 0.5 cm piece of the colon was removed and processed for histology. Small samples of the pieces were processed for electron microscopy, and the remaining colon was used for determining levels of bacterial colonization. Samples were fixed in 10% formaldehyde, wax embedded and stained with hematoxylin and eosin (H&E) (Bancroft and Gamble, 2008).

Assay for colonization

Following opening of the large intestine and removal of samples for histology, the remaining intestine was homogenized mechanically by using a colworth stomacher 80. Serial dilutions were plated on L agar plates containing 100 $\mu\text{g/ml}$ of nalidixic acid and colony forming unit (CFU) in each sample was determined by direct counting.

Results

The isolated *E. coli* O157:H7 could produce both stx1 and stx2 (data not shown).

Effect of hyperimmune LPS antiserum on adherence of *E. coli* O157:H7 to HEP-2 cells and colon of mice

Figures 1a and 1b show untreated HEP-2 cells (without any bacteria and antiserum)

and adherence of bacteria to HEP-2 cells in the group infected with *E. coli* O157:H7. The antiserum raised in rabbit blocked adherence of isolated *E. coli* O157:H7 to HEP-2 cells in a dilution-dependent manner when preincubated with the bacteria for 30 min prior to the infection of the monolayer (Fig. 1c). At a 1:1280 dilution of the antiserum, blocking of bacterial adherence was observed. As the dilution of antiserum increases, partial blocking of bacterial adherence to HEP-2 cells was seen.

The ability of hyper immune LPS antiserum to inhibit the adherence of *E. coli* O157:H7 to colon was examined. Titers 1:10 to 1:640 inhibit the adherence of *E. coli* to the colon. Inhibition in 1:640 was comparable to 1:1280 titer in HEP-2 cells.

SEM of adhesion of *E. coli* O157:H7 to HEP-2 cells

Untreated HEP-2 cells were seen in SEM (Fig. 2a). Bacteria turned out to be adhering to the cells at all time points. *Escherichia coli* O157:H7 showed the filaments formation in HEP-2 cells after 2-3 h which were completed in 6 h (Fig. 2b).

TEM adhesion of *E. coli* O157:H7 to colon tissue

TEM of the colon of the mice infected with this bacterium showed the presence of bacteria adhering to the microvilli (Fig. 3a). No bacteria were identified in mice challenged with *E. coli* O157:H7 and antiserum in 1:640 dilution (Fig. 3b).

Histological examination of the colon

A clear macroscopic change was not evident in colon at day 15 postchallenge. The Colons of mice without challenging by bacteria showed normal histological features (Fig. 4a), while mice inoculated with *E. coli* O157:H7 showed neutrophile, lymphocyte and bacteria inside the lumen (Fig. 4b).

Colonic colonization of mice challenged with *E. coli* O157:H7

The level of colonic colonization (and histological examination) was routinely tested at day 15 of the postchallenge. The mice infected with *E. coli* O157:H7 were colonized as determined by the number of

bacteria recovered from the large intestine. Bacteria were isolated from 2 of the 10 mice infected with bacteria and antiserum. These 2 mice were poorly colonized (Table 1). No bacteria were isolated from mice challenged with PBS.

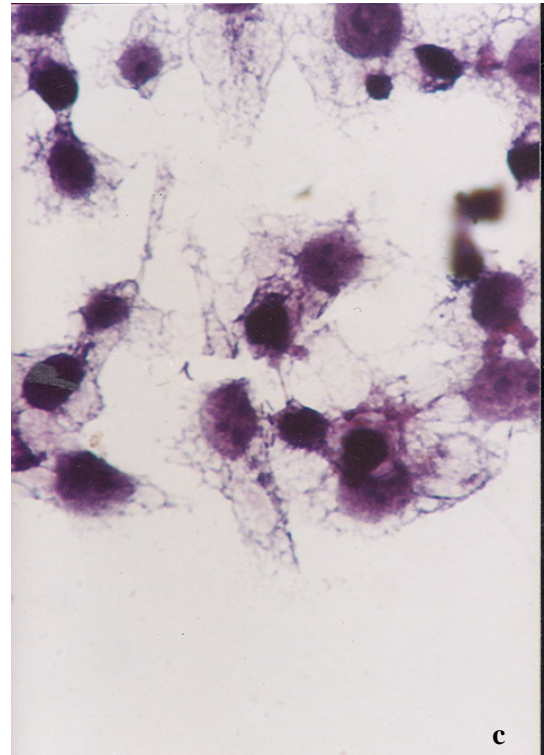
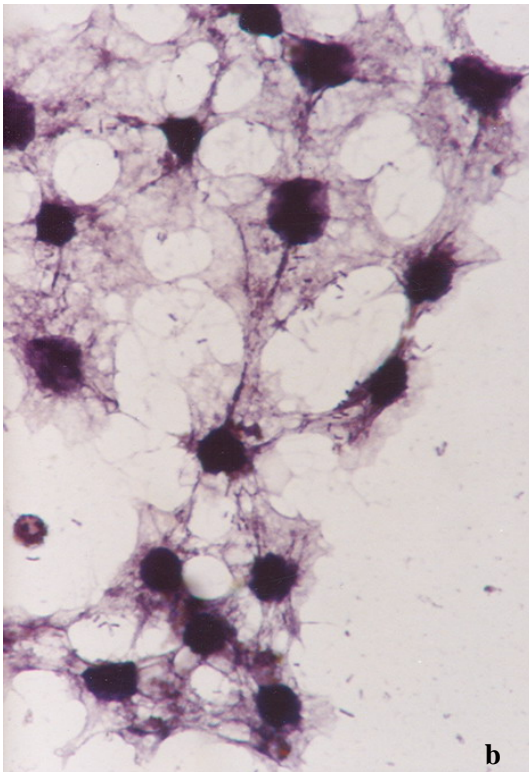
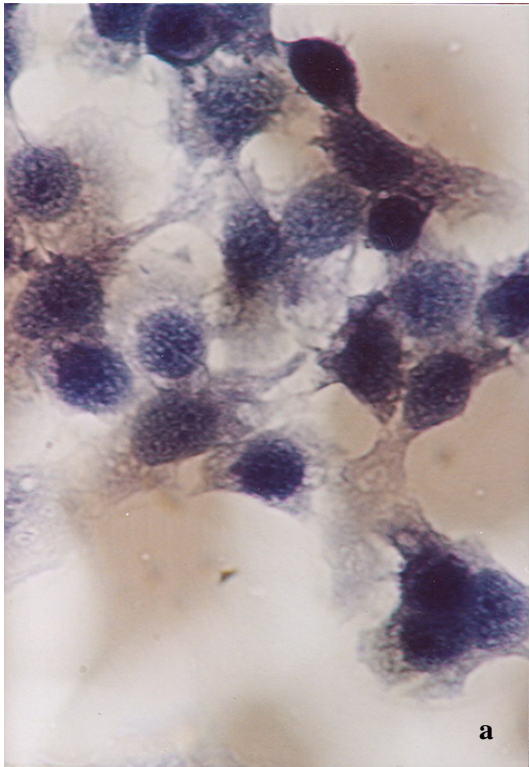


Fig. 1: HEP-2 cells were incubated at 37°C in 5% CO₂ for 72 h in the presence of medium alone or bacteria. Cells were fixed and stained prior to microscopy. (a) Untreated HEP-2 cells, (b) HEP-2 cells incubated with *E. coli* O157:H7 after 3 h and (c) Blocked adherence of isolated *E. coli* O157:H7 to HEP-2 cells in presence of antibody dilution of 1:1024 for 30 min prior to the infection of the monolayer. Cells were photographed with the (magnification, ×1000)

Table 1: Colonic colonization of mice by *E. coli* O157:H7 and *E. coli* O157:H7-antiserum

Mouse No.	CFU/mg of tissue (<i>E. coli</i> O157:H7)	CFU/mg of tissue (<i>E. coli</i> O157:H7- antiserum)
1	7×10 ⁴	0
2	8×10 ³	0
3	1.7×10 ²	0
4	3.5×10 ³	0
5	8.2×10 ²	0
6	6.1×10 ²	0
7	2.6×10 ⁰	0
8	1.4×10 ¹	0
9	6.3×10 ²	0.7×10 ¹
10	8.9×10 ³	0.5×10 ¹

*Mouse mortality following challenge with *E. coli* O157:H7*

By day 15, 7 of 10 mice challenged with *E. coli* O157:H7 died as a result of infection. At this stage, 3 surviving mice were removed for the histological examination and determination of the level of colonic

colonization. No death was seen for the mice challenged with bacteria-antiserum and PBS.

Discussion

Adherence of enteric *E. coli* pathogen to intestinal epithelium plays a crucial role in the pathogenesis of diarrheal diseases because it promotes bacterial colonization

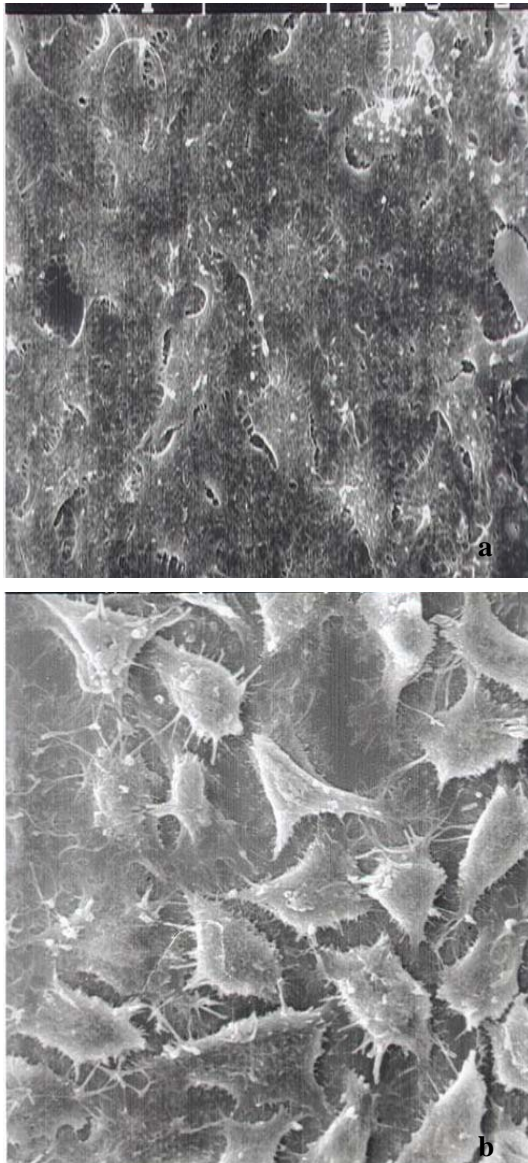


Fig. 2: Scanning electron microscope micrograph of adherence of *E. coli* O157:H7 to cell culture was studied by using HEP-2 cells grown on glass coverslips. At time exposure cells were seen. (a) HEP-2 cells (control) and (b) Adhered *E. coli* O157:H7 to HEP-2 cell which shows the ability of these bacteria to attach and form filaments to HEP-2 cells

and enhances delivery of bacterial exoproducts to the epithelium. In animal models, *E. coli* O157:H7 have been shown to adhere to the apical surface of enterocytes

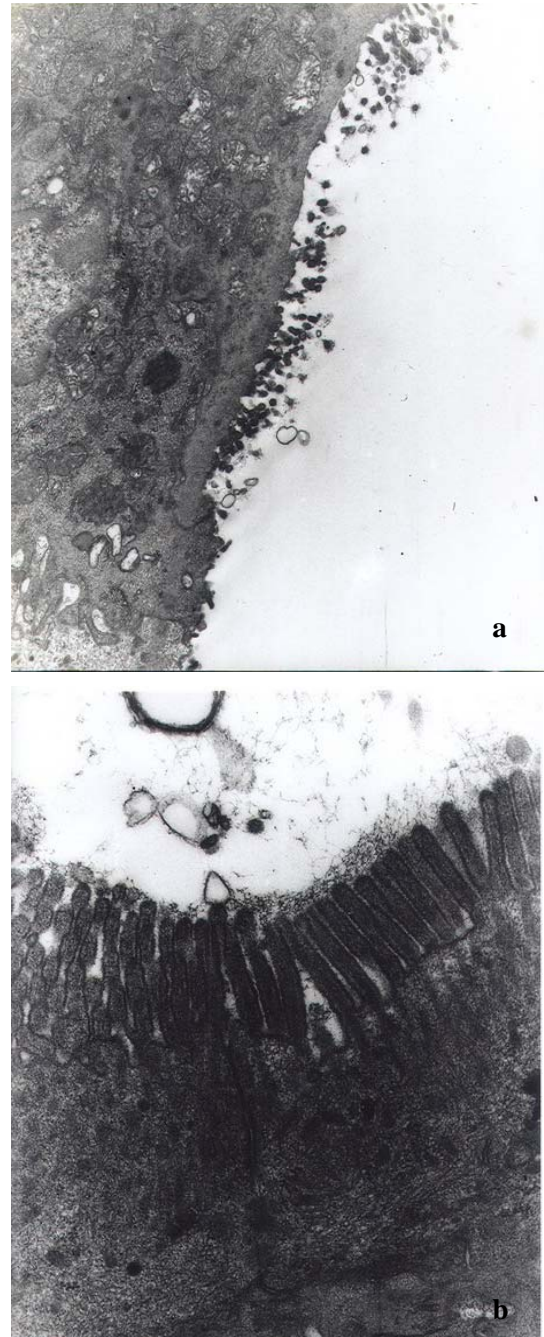


Fig. 3: Transmission electron micrograph of bacterial adherence to the mouse intestine epithelium at day 15 post-infection. (a) Bacterial attachment with microvillous effacement on surface of epithelial cell (magnification, $\times 7000$) and (b) Mouse with challenging by bacteria-antiserum showing normal microvilli on surface epithelium (magnification, $\times 20000$)

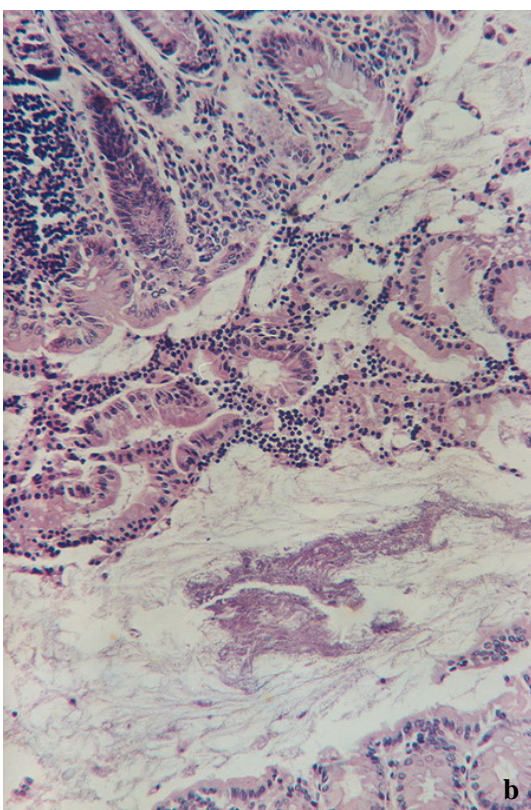
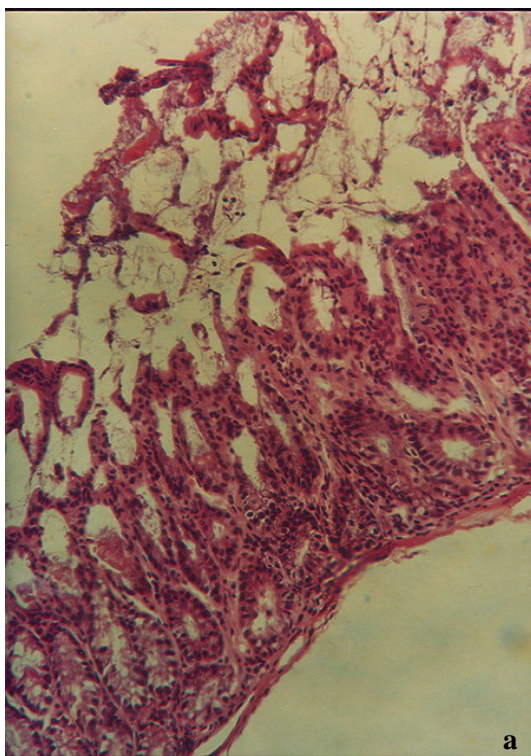


Fig. 4: Colon of mouse without challenging by bacteria showing normal histological features (magnification, $\times 200$) (a), and colon of mouse inoculated with *E. coli* O157:H7 showing presence of neutrophils, lymphocytes and bacteria inside the lumen (magnification $\times 400$) (b) (H&E)

by the A/E lesions (Moon *et al.*, 1983). This type of adherence is sufficient to produce a diarrheal response in gnotobiotic piglets independent of verotoxin production (Tzipori *et al.*, 1987).

It has been shown that the A/E lesion produced by both EPEC and EHEC is associated with actin polymerization in the cytosol immediately beneath the area of bacterial attachment (Knutton *et al.*, 1989; Cockerill III *et al.*, 1996). *Escherichia coli* O157:H7 can survive in bovine faeces for a long time, especially at 15°C (Fukushima *et al.*, 1999). Study about antiserum against intimin shows that this antiserum blocks binding of *E. coli* O157:H7 to cultured epithelial cells. Sinclair and O'Brien (2002) showed that polyclonal antinucleolin sera were able to block and decrease the adherence of *E. coli* O157:H7 to HEP-2 cells. The initial adherence is manifested as the local formation of bacterial micro colonies, called localized adherence (LA), in HEP-2 and other mammalian cell lines. McKee and O'Brien (1995) examine the interactions of *E. coli* O157:H7 strains with human ileocecal (HCT-8) epithelial cells and HEP-2 cells. Knutton *et al.* (1989) shows that EHEC cells adhere to HEP-2 cells in a localized pattern. Konadu *et al.* (1994, 1998) investigate *in vitro* killing assay by using serum obtained from mice and human vaccinated parenterally with protein-conjugated O157 antigen. They have speculated that specific IgG against this antigen, secreted into intestinal mucosa might confer protective immunity against *E. coli* O157:H7, but another research reported that no specific IgG was detected in the faeces of mice in response to orogastric inocula of *E. coli* O157:H7 (Conlan and Perry, 1998). Moradi Bidhendi *et al.* (2007) showed that antiserum against uropathogenic *E. coli* could block attachment of bacteria to the HEP-2 cell in 1:1024 titer and in the tissue of the bladder of mice, 1:512 titer of this antiserum could block attachment.

Our study proves that antibodies can afford protection against bacterial colonization. The ability of the isolated *E. coli* from cattle source to invade eukaryotic cells *in vitro* and *in vivo* was demonstrated by cell culture, histology and electron

microscopy. In our study, the isolated *E. coli* O157:H7 from cattle seems to possess the ability to invade eukaryotic cells and its hyper immune antibody can totally block EHEC adherence to HEP-2 cell in a 1:1280 titer. *In vivo* study shows that 1:640 titers can inhibit adherence of bacteria to the intestine of mice, and the histology of intestine tissue confirms our results. The difference between *in vivo* and *in vitro* titrations for blocking attachment depends on these two different conditions.

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