Travelan as a broad Spectrum anti-bacterial

1. INTRODUCTION

Travelan is harvested from the first milking of dairy cows that have been immunized with a killed bacterial extract to produce high levels of specific antibodies against selected surface antigens from the most common strains of Enterotoxigenic *E. coli* (ETEC). This antigen extract is produced by non-specific mechanical shearing of the bacteria and predominantly contains three important pathogenic and antigenic determinants of ETEC, namely lipopolysaccharide (LPS), flagella, and colonisation factor antigen (CFA). These antigens are important for ETEC outer membrane stability and host immune evasion, motility, and host-cell adherence, respectively.

LPS are large molecules, consisting of a lipid and a polysaccharide joined by a covalent bond, which are found in the outer membrane of Gram-negative bacteria. LPS are endotoxins which elicit strong immune responses in animals and humans, particularly via Toll-like Receptor 4 on cells, and trigger the innate and cell mediated immune system. LPS has three domains: the Lipid A portion which is anchored in the bacterial cell membrane, the core oligosaccharide which contains the inner core attached to the lipid A and the outer core exposed on the cell surface, and the O-polysaccharide which is composed of O-oligosaccharide chains. Antibodies produced against the O-polysaccharide of LPS are serotype specific, whilst those produced against the core and lipid A region can be cross-reactive between bacterial species. Flagellin is a protein that is the principal substituent of bacterial flagellum and is present in large amounts on nearly all flagellated bacteria. Flagellin is also a potent activator of the immune system of the gut via Toll-like Receptor 5.

Evidence presented in this section demonstrates that the antibodies in Travelan has high binding activity against both the O-polysaccharide and the lipid A core region of LPS of gram-negative bacteria. These antibodies recognize LPS from all ETEC strains in the vaccine, as well as other ETEC serotypes and other Gram Negative bacteria. Antibodies in Travelan have also been shown to bind and agglutinate ETEC and prevent adherence of ETEC to gut epithelium *in vitro* and bacterial colonization *in vivo.* Flagella-specific antibodies present in Travelan are able to bind flagellin and reduce motility of ETEC strains and adherence to epithelium.

2. SUMMARY OF PRECLINICAL EXPERIMENTS:

1. Travelan contains anti-LPS antibodies to the thirteen Enterotoxigenic *Escherichia coli* (ETEC) serotypes included in the vaccine [see results in section 3.1]. The antibody response to these individual serotypes was analysed by Western blot with purified LPS. Blots were probed with Travelan hyperimmune colostrum [batch TRV001], non-immune colostrum and individual cow colostrum. The samples selected for the latter ranged from cows with low, moderate and high total antibody responses to the ETEC vaccine determined by ELISA.
   - The Travelan TRV001 blot indicated that anti-LPS antibodies are present against both the O polysaccharide and lipid A core region of all the serotypes included in the ETEC vaccine.
   - The non-immune colostrum blot indicated very low levels of anti-LPS antibodies present. These antibodies are most likely cross-reactive from exposure to *E. coli* in the general farm environment, other than those in the ETEC vaccine.
   - Individual cow responses were variable. Some serotypes are potentially more antigenic than others, as the majority of cows screened produced a response whereas others did not.

2. Travelan contains anti-LPS antibodies that cross-react with LPS from other ETEC and other Gram negative bacteria [see results in sections 3.2 and 3.3].
   - Anti-LPS antibodies present in Travelan batch TRV001 cross react with both lipid A core and O-polysaccharide regions of ETEC serotypes not included in the ETEC vaccine.
   - Anti-LPS antibodies present in Travelan batch TRV001 also cross react with both lipid A core and O-polysaccharide regions of other Gram negative bacteria.

3. Travelan antibodies are able to bind and agglutinate ETEC bacteria [see results in section 3.4].
   - Hyperimmune colostrum batch CT99 was able to agglutinate ETEC strain H10407 (O78) at a minimum concentration of 0.6 mg/ml.
   - Agglutination of the bacteria by specific antibodies present in the colostrum may prevent attachment of the bacteria to the small intestine in humans.

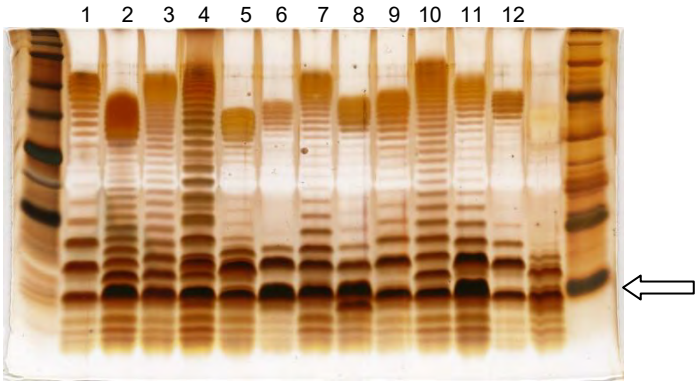
4. Travelan antibodies are able to prevent adherence of ETEC to Caco-2 cell line in *in vitro* assays [see results in section 3.5].
   - The presence of affinity-purified antibody at 500 and 50 µg/ml significantly reduces adherence of ETEC serotype O78 (strain H10407) to Caco-2 cells, used as a model of the gut epithelium. This may be occurring as a consequence of blocking surface expressed bacterial proteins involved with attachment.
   - Travelan batch CT99 colostrum was also able to inhibit adherence of H10407 to Caco-2 cells at the highest concentration tested.

5. Flagella-specific antibodies present in Travelan are able to interrupt two important E. coli pathogenic determinants, namely bacterial motility and adherence to host-cells [see results in section 3.6]. IgG purified from Travelan colostrum:
   - Significantly reduces adherence of CFA/I producing ETEC strains to a cell-line that mimics the human small intestinal epithelium.
   - Reduces the motility of ETEC strains through soft agar.
   - Binds to both the bacterial surface and flagella.
   - Has substantially greater reactivity against purified ETEC flagella antigen than IgG purified from non-immune colostrum powder.

6. A passive immunity study using a mouse respiratory model showed reduction of ETEC load with homologous and heterologous LPS antibodies [see results in section 3.7].
   - It was shown that serotype specific LPS antibody given intranasally prior to ETEC inoculation can reduce bacterial colonisation in the mouse lung.
   - Purified CT99 IgG, H10407 LPS IgG, H10407 LPS F(ab) and Salmonella LPS F(ab) fragments from anti-LPS colostrum batch CT99 were all significantly better at reducing infection compared to the PBS control.

3. PRECLINICAL EXPERIMENTAL RESULTS

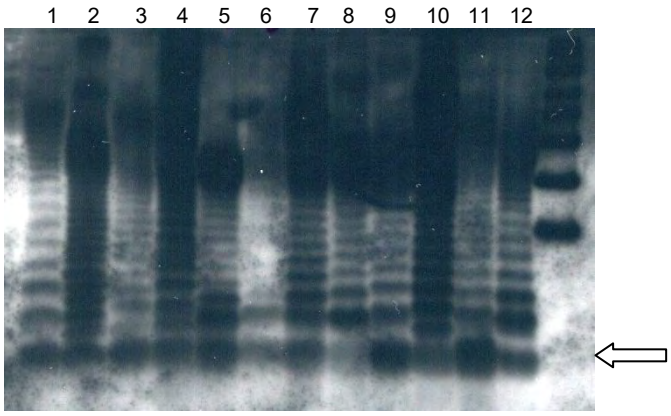
3.1 Travelan antibodies binding to LPS extracted from ETEC serotypes in vaccine



A. The above photo shows LPS extracted from ETEC strains in Immuron's proprietary ETEC vaccine, run on 15% Tris-tricine SDS-PAGE and stained using LPS specific silver stain. Photo shows O-polysaccharide chains as ladders and lipid A core region (arrow).

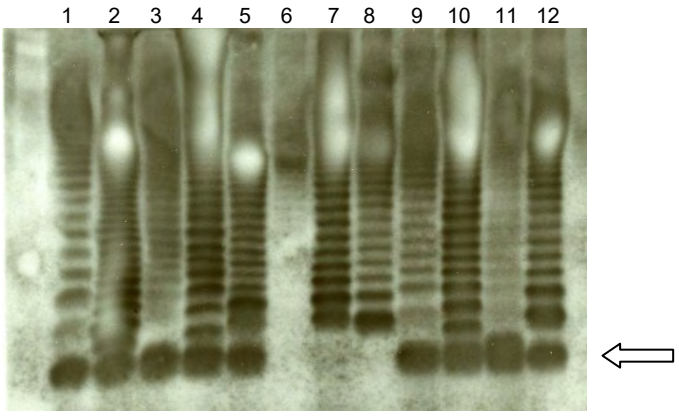
Lane 1 - ETEC strain B2C [serotype O6]
2 - ETEC strain C55 3/3c3 [serotype O8]
3 - ETEC strain PE 595 [serotype O15]
4 - ETEC strain E11881A [serotype O25]
5 - ETEC strain C1064-77 [serotype O27]
6 - ETEC strain PE 672 [serotype O63]
7 - ETEC strain E20738/0 [serotype O114]
8 - ETEC strain PE 724 [serotype O115]
9 - ETEC strain EI 37-2 [serotype O128]
10 - ETEC strain B7A [serotype O148]
11 - ETEC strain E8772/0 [serotype O153]
12 - ETEC strain PE 768 [serotype O159]

ETEC strain H10407 [O78:H11] was not included in this study as it was administered as a single vaccine



B. The above photo shows western transfer to PVDF membrane and blotted with Travelan batch TRV001, made from a pool of colostrum from over 1000 cows. This photo shows binding of anti-LPS antibodies against both the O-polysaccharide

ladders and arrowed lipid A core region of all the serotypes included in the ETEC vaccine.



C. The above photo shows Western transfer to PDVF membrane and blotted with colostrum from single hyper-immunized cow 12508. Cow 12508 has produced antibodies against the O chain regions of the ETEC serotypes. It has also produced antibodies against the lipid A core regions of all serotypes, excluding three (O63, O114 & O115 – see arrowed region).

Summary of Results (section 3.1):

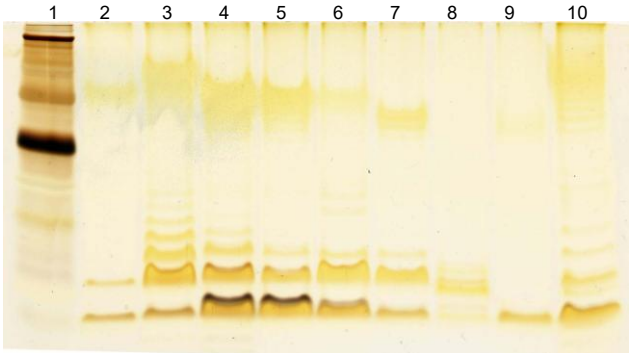
Travelan contains anti-LPS antibodies to the thirteen Enterotoxigenic *Escherichia coli* (ETEC) serotypes included in Immuron's proprietary vaccine:

- The Travelan TRV001 blot indicated that anti-LPS antibodies are present against both the O polysaccharide and lipid A core region of all the serotypes included in the ETEC vaccine.
- The non-immune colostrum blot indicated very low levels of anti-LPS antibodies present. These antibodies are most likely cross-reactive from exposure to *E. coli* in the general farm environment, other than those in the ETEC vaccine.
- Individual cow responses were variable. Some serotypes are potentially more antigenic than others, as the majority of cows screened produced a response whereas others did not.

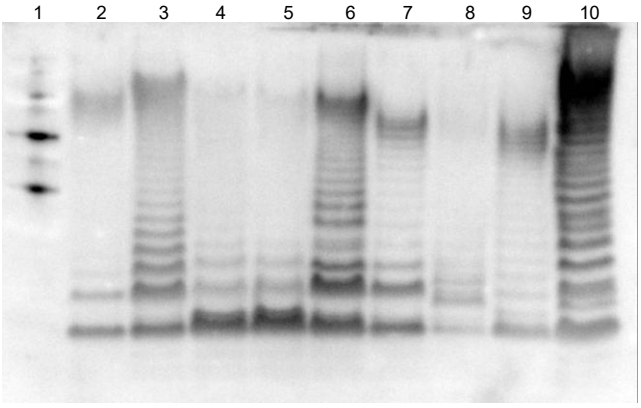
3.2 Travelan anti-LPS antibody binding to LPS from other ETEC serotypes not in vaccine

A. Silver stained 15% Tris-tricine gel showing LPS extracts from ETEC serotypes not included in Travelan vaccine.

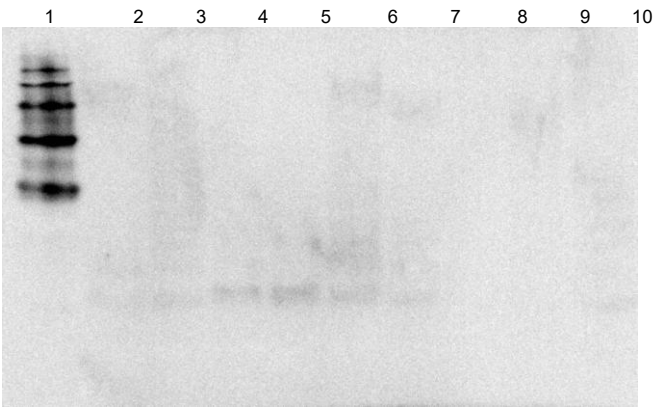
Lane 1 - Protein standard
2 - ETEC strain M452C1 [serotype O20:H-]
3 - ETEC strain T0225-C4 [O75:H4]
4 - ETEC strain 83-552 [O126:H-]
5 - ETEC strain G33 [O126:H12]
6 - ETEC strain M145C2 [O128:H(NT)]
7 - ETEC strain E23477/0/A [O139:H25]
8 - ETEC strain ND782 [O141:H4]
9 - ETEC strain ND748 [O149:H10]
10 - ETEC strain E11881A [O25:H42] (+ control)



B. Western blot of above LPS extracts from ETEC serotypes, using Travelan batch TRV001 as primary antibody. Anti-LPS antibodies present in Travelan batch TRV001 cross react with both lipid A core and O polysaccharide regions of ETEC serotypes not present in the Travelan vaccine.

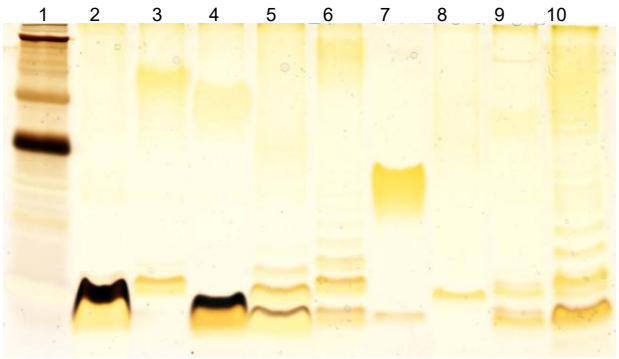


C.Western blot of same LPS extracts from ETEC serotypes, using non-immune colostrum as source of primary antibody. Non-immune colostrum contains very low levels of anti-LPS antibodies that cross react with ETEC LPS.



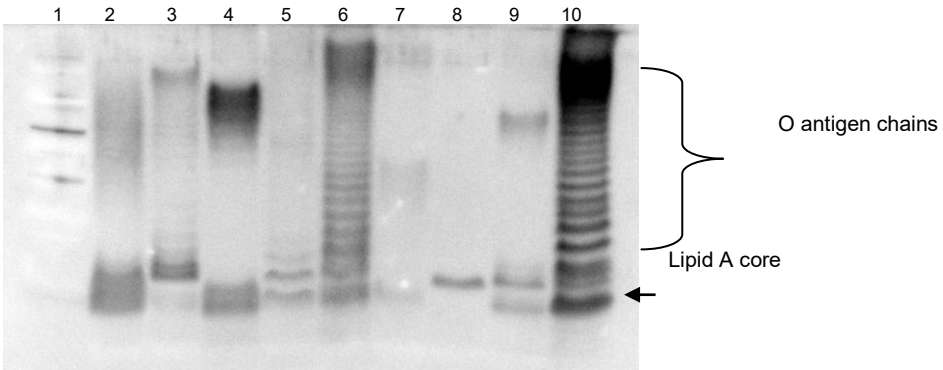
3.3 Travelan anti-LPS antibody binding to LPS from selected Gram negative bacteria

A. Silver stained 15% Tris-tricine gel of LPS extracts from other gram negative bacteria.

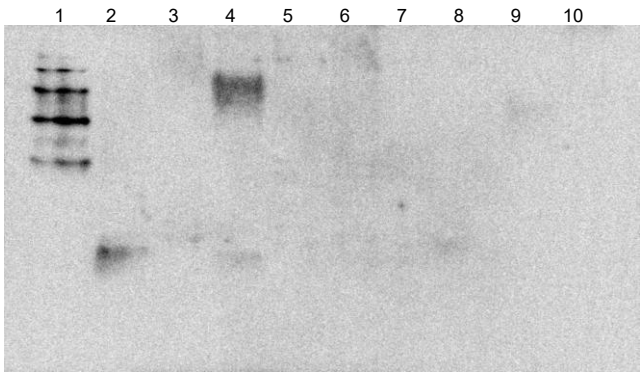


Lane 1 - Protein standard
2 - Enterobacter aerogenes strain ATCC 13048
3 - Enteropathogenic Escherichia coli strain E2348/69
4 - Klebsiella pneumoniae strain ATCC 26
5 - Pseudomonas aeruginosa strain ATCC 27853
6 - Salmonella typhimurium strain ATCC 14028
7 - Vibrio cholerae strain 6239
8 - Yersinia enterocolitica strain 67R
9 - Citrobacter rodentium strain DSB100
10 - ETEC strain E11881A [O25:H42] (+ control)

B. Western blot of above LPS extracts from other gram negative bacteria, using Travelan batch TRV001. Blot shows that Anti-LPS antibodies present in Travelan batch TRV001 cross react with both lipid A core and O polysaccharide regions of the above listed Gram negative bacteria.



C. Western blot of above LPS extracts from other gram negative bacteria, using non-immune colostrum. Blot shows that Non-immune colostrum contains only a very small amount of anti-LPS antibody compared to Travelan hyperimmune colostrum.



Summary of Results [sections 3.2 and 3.3]:

Travelan contains anti-LPS antibodies that cross-react with LPS from other ETEC and other Gram negative bacteria:

- Anti-LPS antibodies present in Travelan batch TRV001 cross react with both lipid A core and O-polysaccharide regions of ETEC serotypes not included in the ETEC vaccine.
- Anti-LPS antibodies present in Travelan batch TRV001 also cross react with both lipid A core and O-polysaccharide regions of other Gram negative bacteria.

3.4 Travelan anti-LPS antibody is able to bind and agglutinate ETEC bacteria

Experiments showing agglutination of Enterotoxigenic *Escherichia coli* (ETEC) using CT99 single strain hyperimmune colostrum:

Materials and Methods
Luria Agar
10 g/L Tryptone
10 g/L NaCl
5 g/L Yeast Extract
15 g/L Bacto Agar

Dissolve components in 1 L dH_2O, autoclave at 121 °C for 20 minutes.

ETEC Strains
H10407: CFA positive
H10407: Nataro strain
H10407: E1
H10407: ST, LT, CFA negative
M452C1: CFA negative
ETEC strains were grown on Luria agar plates overnight at 37 °C.

Hyperimmune Colostrum
CT99 anti-ETEC colostrum was made up as a stock solution in PBS at a concentration of 10 mg/ml. The suspension was dissolved overnight at 4 °C. Serial dilutions were made in PBS for use in the agglutination assay.

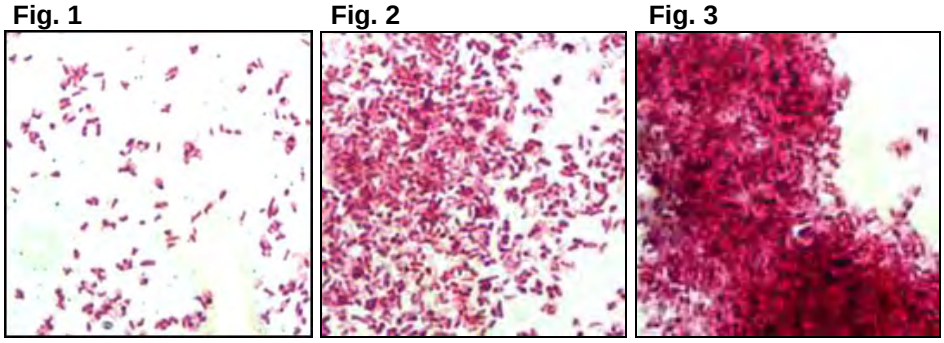
Slide Agglutination Assay
A thick suspension of each bacterial strain was made in 20 µl of PBS direct onto a glass microscope slide. The bacteria were taken direct from the agar plate. 20 µl of each dilution of CT99 colostrum was added to a bacterial suspension on the microscope slide and mixed with a pipette tip. A positive result was scored as agglutination occurring within a five minute time period. Suspensions were also Gram stained for visualisation of bacterial agglutination by microscopy.

Gram Stain
Bacterial suspensions +/- CT99 colostrum (from slide agglutination assay) were diluted 1:20 in a total volume of 20µl on a glass microscope slide allowed to dry and heat fixed.

1. Stain with crystal violet for 1 minute
2. Pour off crystal violet and stain with iodine for 1 minute.
3. Decolourise with Acetone and wash immediately with dH_2O.
4. Counterstain for Gram negative bacteria with Neutral Red for 1 minute.
5. Wash the slide with dH_2O and allow to dry before visualisation by microscopy.

Using 100x objective with oil immersion the bacteria can be seen either as single rods or agglutinated by the CT99 colostrum.

Fig. 1 **Fig. 2** **Fig. 3**



Gram stain of H10407 with and without CT99 colostrum; Figure 1: 0 mg/ml CT99 colostrum, Figure 2: 1 mg/ml CT99 colostrum, Figure 3: 5 mg/ml CT99 colostrum.

Results

Table 1: Agglutination assay using CT99 at a concentration of 10 mg/ml

ETEC Strains	**+/- Agglutination**
H10407 CFA+	+
H10407 E1	+
H10407 Nataro	-
H10407 $ST^-/LT^-/CFA^-$	+
M452C1 CFA^-	+

Table 2: Agglutination assay using H10407 CFA + strain with varying concentrations of CT99 colostrum.

CT99 [mg/ml]	**+/- Agglutination / Time to Agglutinate**
10	+ / 1 sec
5	+ / 20 sec
2.5	+ / 30 sec
1.25	+ / 60 sec
1.0	+ / 4 min
0.9	+ / 4 min
0.8	+ / 4 min
0.7	+ / 4 min
0.625	+ / 4 min 20 sec
0.6	+ / 4 min 20 sec
0.3125	-
0.15625	-
0.078125	-
0	-

Summary of Results (section 3.4):

Travelan antibodies are able to bind and agglutinate ETEC bacteria:

- Hyperimmune colostrum batch CT99 was able to agglutinate ETEC strain H10407 (O78) at a minimum concentration of 0.6 mg/ml.
- Agglutination of the bacteria by specific antibodies present in the colostrum may prevent attachment of the bacteria to the small intestine in humans.

3.5 Serotype specific anti-LPS antibodies inhibit adherence of ETEC to Caco-2 cells in vitro

A. <u>Inhibition of adherence of ETEC to Caco-2 cell line in the presence of affinity purified H10407 LPS antibody and CT99 hyperimmune colostrum</u>:

Materials and Methods

<u>Caco-2 Cell Line</u>

Cells were routinely maintained in DMEM supplemented with 20 % FCS, 20 mM HEPES pH 7.5 and 2 mM glutamine. For use in the assay DMEM supplemented with 1 % FCS, 20 mM HEPES, 2mM glutamine and 0.5 % Mannose. Cells were seeded at a density of 1 x 10^5 cells/well in a 24 well tissue culture tray. Cells were grown until 12 days post confluent for use in the assay. Media was changed daily after cells reached 100 % confluence.

<u>Affinity Purified LPS Ab and CT99 colostrum</u>

LPS antibody was affinity purified from CT99 hyperimmune colostrum. Briefly, purified LPS from ETEC strain H10407 was coupled to an epoxy-sepharose 6B column. Whey preparation from CT99 was applied to the column. Affinity purified LPS antibody was eluted and concentrated for use in the adherence/inhibition assay. A stock solution at 2 mg/ml was kept at 4 °C.

CT99 hyperimmune colostrum was made up as a stock solution in PBS at a concentration of 5 mg/ml. The suspension was dissolved overnight at 4 °C and filter sterilised using a 0.2 µm filter to remove any contaminants and particulate matter. The stock solution was kept at 4°C.

<u>Bacterial Strain and Culture Conditions</u>

ETEC strain H10407 was grown on CFA agar (containing 1 % Casamino Acids, 0.15 % yeast extract, 0.005 % magnesium sulphate, 0.0005 % manganese chloride and 2 % agar) and Luria agar (containing 1 % tryptone, 1 % sodium chloride, 0.5 % yeast extract and 1.5 5 agar) overnight at 37 °C.

<u>Adherence/Inhibition Assay</u>

1. H10407 grown on CFA or LA were scraped off plates and made to a McFarland 0.5 equivalent ~ 1 x 10^8 CFU/ml in PBS.
2. 1 ml aliquots were made into eppendorf tubes, 10 tubes each for CFA and LA grown H10407. The bacterial suspension was centrifuged at 13 000 rpm for five minutes.
3. Pellets were resuspended in assay media with and without affinity purified LPS antibody or CT99 colostrum (see volumes below)

LPS Ab concentrations tested	**Antibody: Bacterial Suspension**
[1] 500 µg/ml	250 µl : 750 µl
[2] 50 µg/ml	25 µl : 975 µl
[3] 5 µg/ml	2.5 µl : 997.5 µl
CT99 concentrations tested	
[1] 500 µg/ml	100 µl : 900 µl
[2] 5 µg/ml	1 µl : 999 µl

4. Caco-2 cells were washed twice with warmed PBS (red) before bacterial suspensions were added.

5. 1 ml of each bacterial suspension with or without LPS Ab or CT99 colostrum was added to each well of the cultured Caco-2 cells.
6. Bacteria and cells were incubated for three hours at 37 °C, 5 % CO_2/air.
7. After incubation wells were washed twice with warmed PBS red.
8. 500 µl of Giemsa buffer was added to each well for two minutes.
   Giemsa Buffer
   33.86 ml of 1 M KH_2PO_4
   32.80 ml of 1 M Na_2HPO_4
   Volume adjusted to 1 L solution autoclaved 121 °C for 20 minutes.
9. Buffer removed and 500 µl 100 % Methanol added to each well for ten minutes.
10. Methanol was removed and 500 µl of 10 % Giemsa stain added to each well for four minutes.

    Giemsa Stain
    10 ml Giemsa stain (BDH)
    90 ml dH_2O
    Filtered through Whatman filter paper
11. Stain removed and wells washed twice with Giemsa buffer.
12. Wells air-dried and cells examined on inverted microscope under phase contrast with 40x objective.

Results

Figure 1-6: H10407 grown on CFA agar
Figure 7-12: H10407 grown on Luria agar


Figure 1: H10407: CFA +ve Control

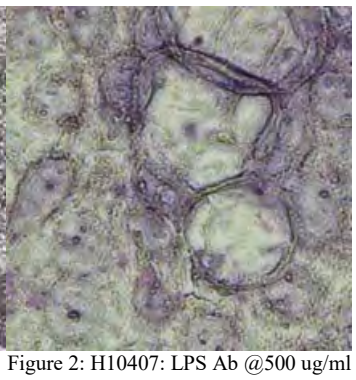


Figure 2: H10407: LPS Ab @500 ug/ml

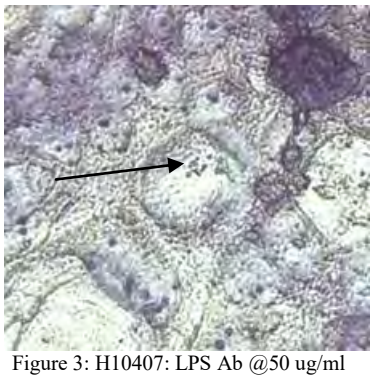


Figure 3: H10407: LPS Ab @50 ug/ml

Arrow: Indicates adherent bacteria →

Figure 1: Adherence of H10407 strain grown on CFA agar, to Caco-2 cells 12 days post confluence. Bacteria preferentially adhering to "domes" of fully differentiated Caco-2 cells.

Figure 2: Inhibition of adherence of H10407 to Caco-2 cells in the presence of 500 µg/ml affinity purified H10407 LPS antibody.

Figure 3: Inhibition of adherence of H10407 to Caco-2 cells in the presence of 50 µg/ml affinity purified LPS antibody. Some bacteria are still adherent in the presence of LPS Ab at 50 µg/ml, these bacteria are adherent to domes.

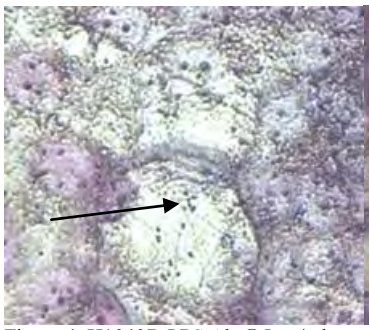


Figure 4: H10407: LPS Ab @5 ug/ml

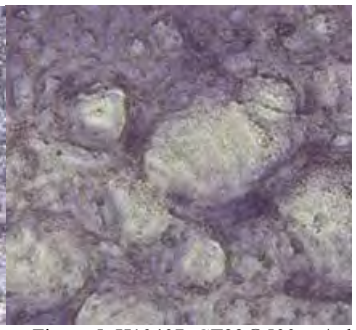


Figure 5: H10407: CT99@500 ug/ml

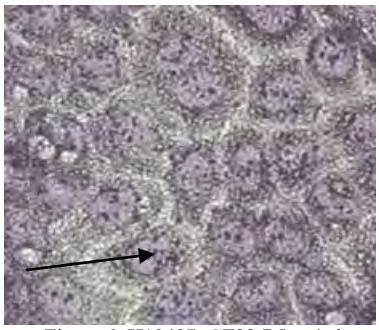


Figure 6: H10407: CT99@5 ug/ml

Arrow: Indicates adherent bacteria →

Figure 4: Limited inhibition of adherence of H10407 to Caco-2 cells in the presence of 5 µg/ml LPS Ab. There are more bacteria present compared with 500 and 50 µg/ml LPS Ab treated wells.

Figure 5: Inhibition of adherence of H10407 to Caco-2 cells in the presence of 500 µg/ml CT99 colostrum.

Figure 6: Limited inhibition of adherence of H10407 to Caco-2 cells in the presence of 5 µg/ml CT99 colostrum. The amount of adherent bacteria present is comparable to H10407 alone grown on CFA agar.

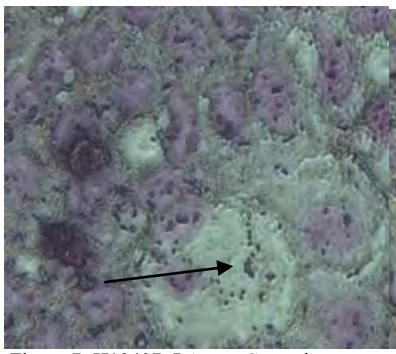


Figure 7: H10407: LA +ve Control


Figure 8: H10407: LPS Ab@500 ug/ml

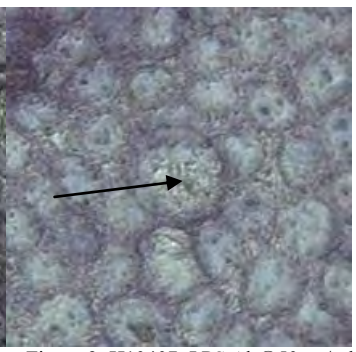


Figure 9: H10407: LPS Ab@50 ug/ml

Arrow: Indicates adherent bacteria →

Figure 7: Adherence of H10407 strain grown on LA, to Caco-2 cells 12 days post confluence. The bacteria do not preferentially adhere to domes when they are grown on Luria agar.

Figure 8: Inhibition of adherence of H10407 to Caco-2 cells in the presence of LPS Ab at 500 µg/ml.

Figure 9: Inhibition of adherence of H10407 to Caco-2 cells in the presence of LPS Ab at 50 µg/ml. Some bacteria still adherent (see Fig 3 for comparison)

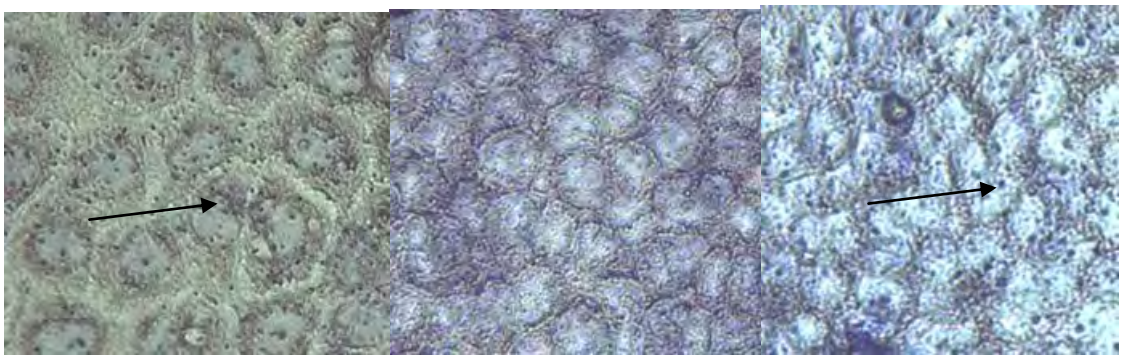


Figure 10: H10407: LPS Ab @5 ug/ml Figure 11: H10407: CT99@500 ug/ml Figure 12: H10407: CT99@5 ug/ml

Arrow: Indicates adherent bacteria →

Figure 10: Limited inhibition of adherence of H10407 to Caco-2 cells in the presence of 5 μg/ml LPS Ab. The inhibition is less that that seen in Fig 4.

Figure 11: Inhibition of adherence of H10407 to Caco-2 cells in the presence of CT99 colostrum at 500 μg/ml.

Figure 12: Adherence of H10407 to Caco-2 cells in the presence of 5 μg/ml CT99 colostrum. There appears to be no inhibition of adherence in this sample.

Conclusions

- The presence of affinity-purified antibody at 500 and 50 μg/ml appears to significantly reduce adherence of H10407 to Caco-2 cells. This may be occurring as a consequence of agglutination of the bacteria in the presence of antibody, thus preventing adherence of bacteria to the cell surface.

- CT99 colostrum was also able to inhibit adherence of H10407 to Caco-2 cells at the highest concentration tested. This also may be due to agglutination of the bacteria, preventing adherence to the cell surface.

B. Serotype specific antibodies to LPS inhibit binding of ETEC to Caco-2 cell line:

Materials & Methods

Colostrum

A Monovalent Travelan: contained antibodies to H10407 (cows vaccinated with ETEC strain H10407 only).

B Polyvalent-2Travelan: contained antibodies to 11 ETEC serotypes including strain H10407.

C Polyvalent -1 Travelan: contained antibodies to 10 ETEC serotypes excluding strain H10407 and including Salmonella.

Antibodies: H10407 and *Salmonella* Typhimurium IgG were affinity purified from monovalent Travelan and polyvalent-2 Travelan respectively. F(ab) fractions were prepared from the affinity purified IgG by papain digestion.

Caco-2 adherence/inhibition assay

For microscopy

1. Caco-2 cells were grown to 15 days post confluence on NUNC Lab Tec® permanox chamber slides.
2. 1 x 10^7 CFU of *E. coli* H10407 was added to each chamber of the Caco-2 cell culture. Antibodies (Travelan IgG, H10407 LPS IgG, F(ab), *Salmonella* LPS IgG and F(ab) used at 250 µg/ml) or PBS were added to the culture and incubated for three hours at 37 ºC in 5 % CO2:air.
3. Cells were washed, stained with Giemsa and dried before visualisation.

For bacterial culture

1. Caco-2 cells were grown to 15 days post confluence in NUNC 24 well tissue culture trays.
2. 7.5 x 10^6 CFU of *E. coli* H10407 was added to each chamber of the Caco-2 cell culture. Antibodies (control whey at 2 mg/ml, P2-Travelan IgG at (a) 0.8 mg/ml, (b) 0.16 mg/ml, and M-Travelan whey at (c) 2.0 mg/ml, (d) 0.4 mg/ml) or PBS were added to the culture and incubated for three hours at 37 ºC in 5 % CO2:air.
3. Cells were washed and treated with 0.1 % digitonin before a plate count was performed. Results expressed as % adherence compared to control whey.

Results

Caco-2 adherence/inhibition assay

Bacterial culture

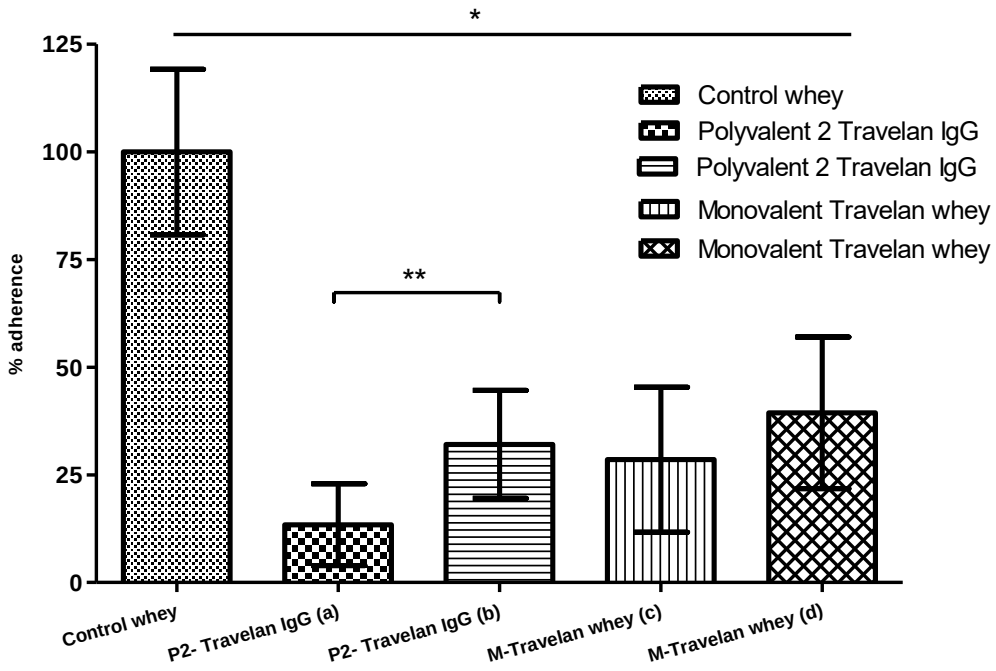


Figure 1: Adherence of ETEC H10407 to Caco-2 cells in the presence of control whey (from unvaccinated cows) at 2 mg/ml, P2-Travelan IgG at **(a)** 0.8 mg/ml, **(b)** 0.16 mg/ml, and M-Travelan whey at **(c)** 2.0 mg/ml, **(d)** 0.4 mg/ml. (* P< 0.001, ** P< 0.05, 1-way ANOVA)

Caco-2 adherence/inhibition assay

Microscopy: Serotype specific antibody

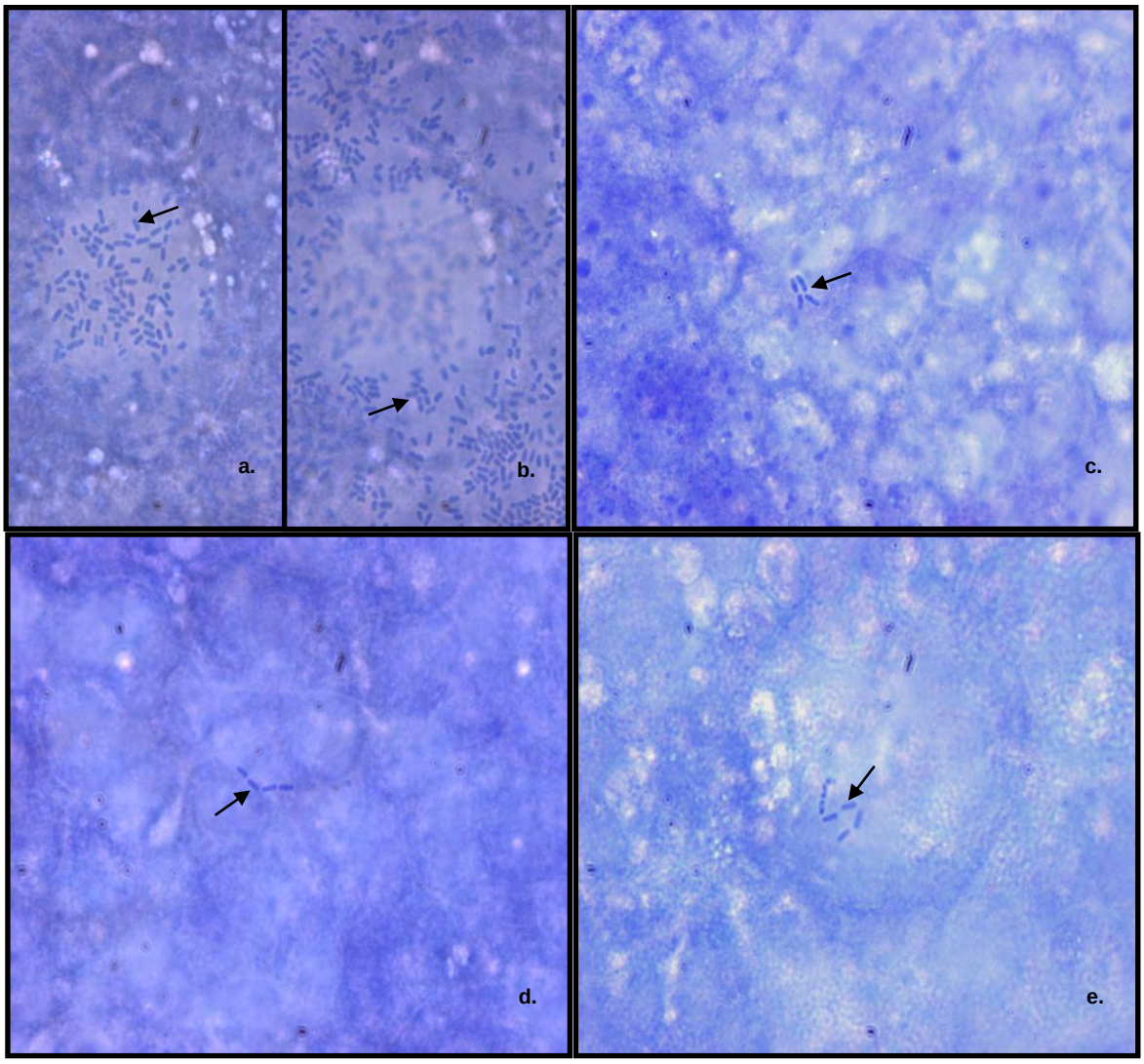


Figure 2: Inhibition of ETEC adherence to Caco-2 cells by specific antibodies. **a.** and **b.** ETEC strain H10407 (O78:H11) adhering to 15-day post-differentiated Caco-2 cells; **c.** with 250 µg/ml monovalent Travelan IgG^{A}; **d.** with 250 µg/ml H10407-LPS IgG, and **e.** with 250 µg/ml H10407-LPS F(ab).

Caco-2 adherence/inhibition assay

Microscopy: Heterotypic antibody

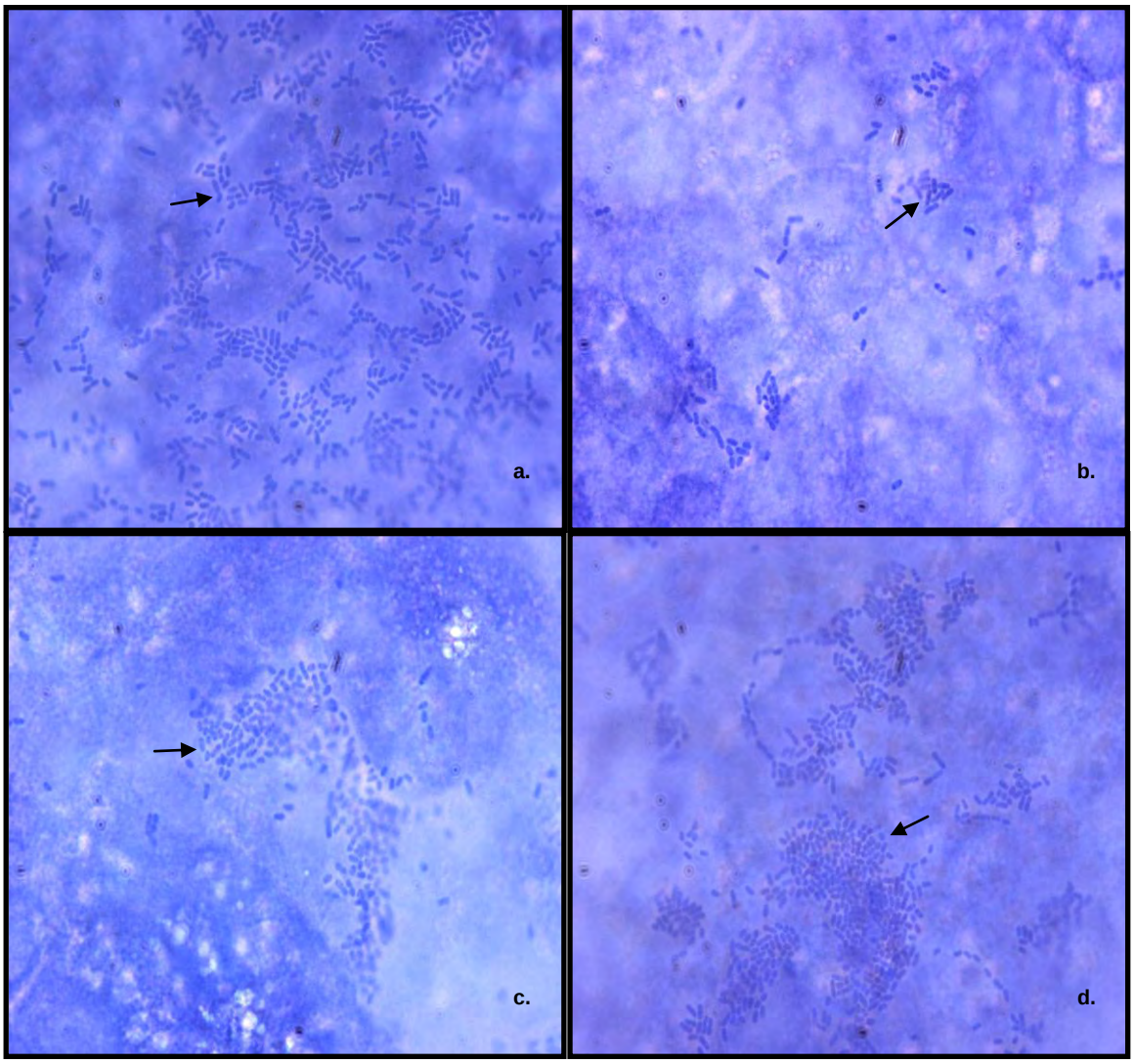


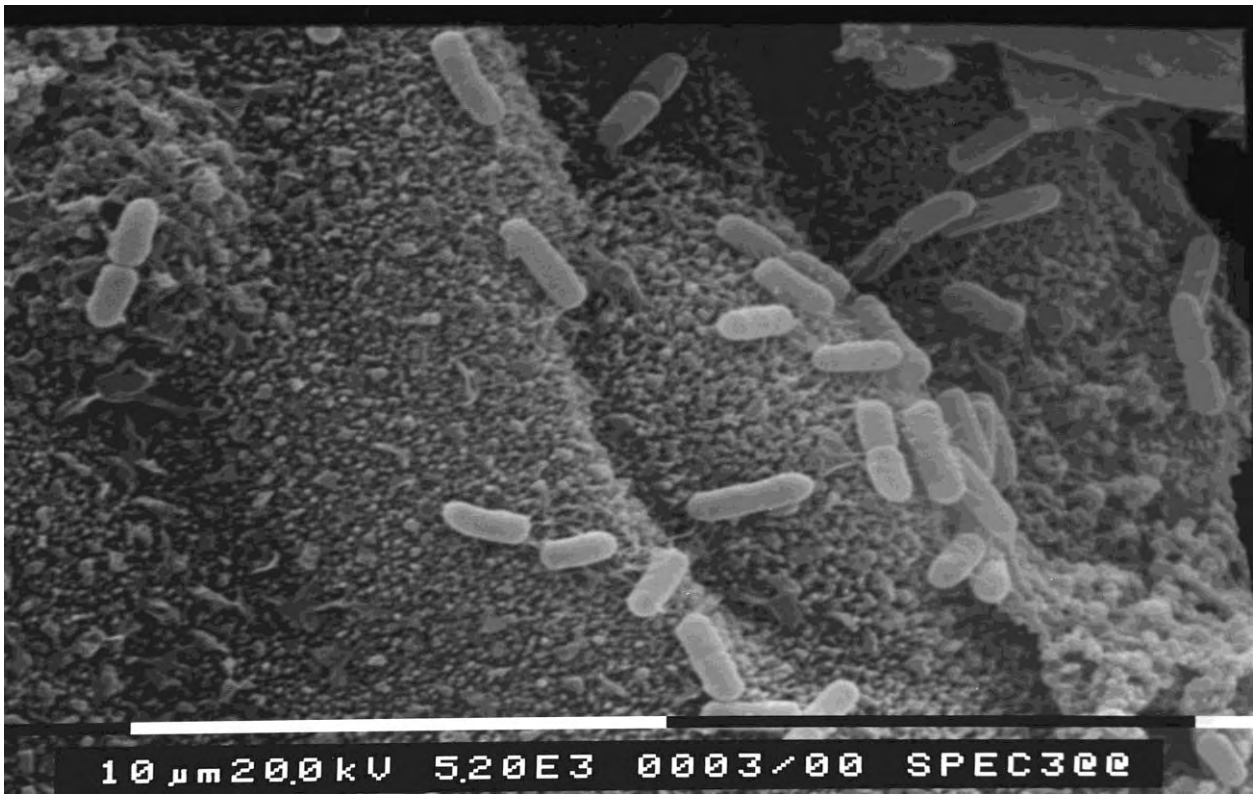
Figure 3: **a.** H10407 adhering to 15 day post-differentiated Caco-2 cells; **b.** with 250 µg/ml polyvalent-1 Travelan IgGC; **c.** with 250 µg/ml Salmonella-LPS IgG, and **d.** with 250 µg/ml Salmonella-LPS F(ab).

Conclusions

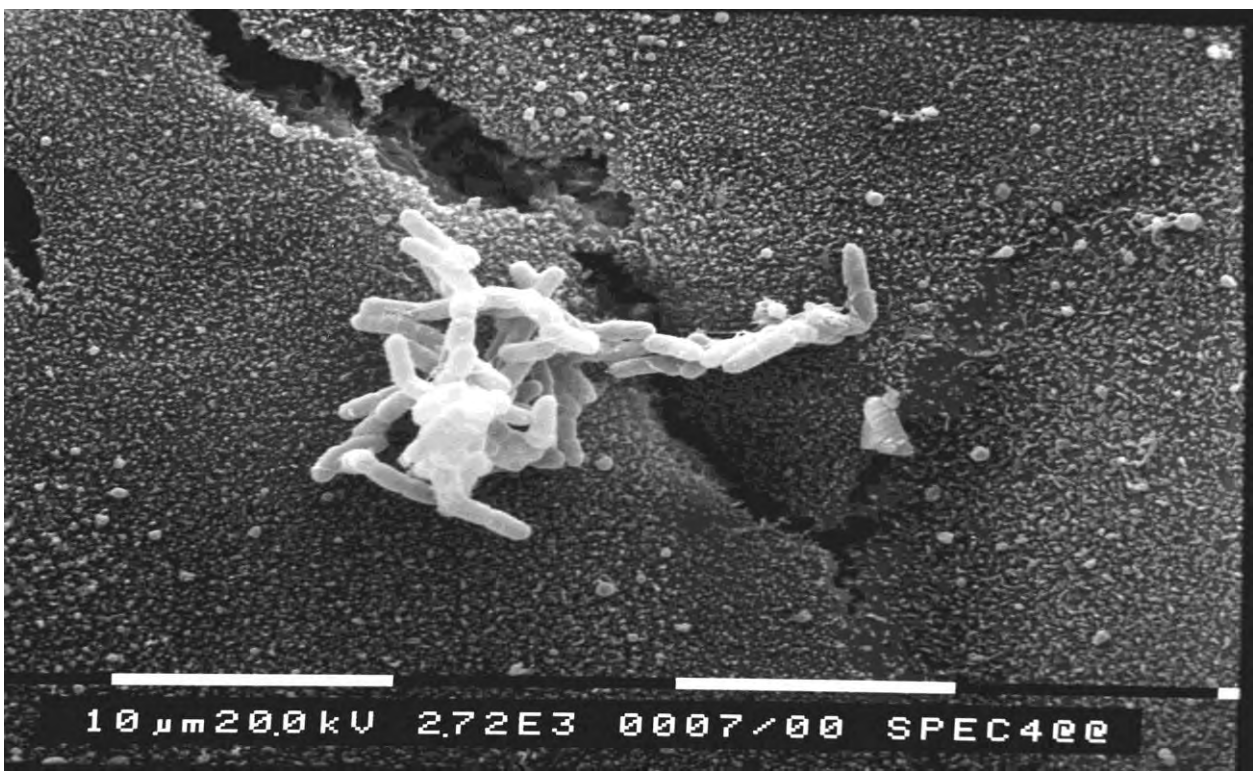
- Serotype specific anti-O antibodies from Travelan inhibited binding of ETEC strain H10407 to Caco-2 cells. F(ab) fragments prepared from these antibodies also inhibited binding, indicating the antibodies did not need to agglutinate the bacteria to mediate their effect.
- Affinity-purified IgG and F(ab)'s directed against the O-antigens of *Salmonella* Typhimurium did not inhibit adherence of ETEC H10407 to Caco-2 cells, except when used at high concentrations.
- Inhibition of adherence of H10407 to Caco-2 cells by Travelan® antibodies was dose dependent.

C. <u>Scanning Electron Micrographs (SEM) showing binding of ETEC to Caco-2 cells with and without anti-LPS antibodies:</u>

(a) ETEC adhering to Caco-2 cell line – without antibodies:



(b) ETEC in the presence of "Travelan" antibodies. Bacteria have agglutinated and very few bacteria found on surface of cells:



Summary of Results (section 3.5):

Travelan antibodies are able to prevent adherence of ETEC to Caco-2 cell line in *in vitro* assays:

- The presence of affinity-purified antibody at 500 and 50 µg/ml significantly reduces adherence of ETEC serotype O78 (strain H10407) to Caco-2 cells, used as a model of the gut epithelium. This may be occurring as a consequence of blocking surface expressed bacterial proteins involved with attachment.
- Travelan batch CT99 colostrum was also able to inhibit adherence of H10407 to Caco-2 cells at the highest concentration tested.

3.6 Travelan antibody binding to Flagellin in ETEC serotypes

A. Detection of Flagellin and Presence of Flagellin Antibodies in Travelan:

Materials & Methods

Bacterial Strains

Human enterotoxigenic *Escherichia coli* strains B7A O148:H28, H10407 O78:H11, E123-7 O128:H21, B2C O6:H16, E11881A O25:H24, E8772/0 O153:H12, human adherent invasive *E. coli* strain LF82 O83:H1, bovine ETEC strain K99, human isolate *E. coli* strain HS and *E. coli* lab strain HB101 were used.

Growth Conditions

All bacterial strains were passaged three times on 0.35 % Luria Bertani (LB) swarm agar, grown at 30 ºC. Bacteria that grew at the outermost edge of swarm were then used as a starter culture for 10 ml LB broths (HB101 showed non-motile phenotype). Broths were grown as a static culture, overnight, at 30 ºC. 10 µl of overnight culture was used to inoculate a fresh 100 ml LB broth, which was grown overnight at 30 ºC. Bacterial motility was checked by wet mount hanging drop, by light microscopy.

Purification of Flagellin

1. Overnight cultures (100 ml) were centrifuged at 3 800 rpm for 30 min at 4 ºC to pellet bacteria. Bacterial pellets were resuspended in a total volume of 1 ml of 150 mM NaCl: 10 mM HCl (pH ~ 1.5) to dissociate flagella from the bacterial surface.

2. The bacterial suspension was transferred to a 2 ml Eppendorf tube and placed on a rotary wheel at top speed, incubated for 1 hour at RT ºC. This suspension was centrifuged at 8 000 xg for 15 mins at 4 ºC.

3. 600 µl of each supernatant was neutralised with the addition of 50 µl of 50 mM Tris: 10 mM NaOH. The neutralised solutions were placed into a 1.5 ml Beckman ultra-centrifuge tube.

4. The supernatants were ultra-centrifuged at 100 000 xg for 90 mins at 4 ºC. Supernatants were carefully removed and discarded after centrifugation; pellets were resuspended in a volume of 100 µl of PBS, overnight at 4 ºC. Flagellin preparations were stored at – 20 ºC before being used.

SDS-PAGE & Western Blot

Flagellin samples were run on 10 % SDS-PAGE (Laemmli buffer system) before being Coomassie stained or transferred to PVDF membrane for Western blot. Western blot was performed using Travelan batch TRV001 at a concentration of 5 mg/ml in PBS. A secondary goat α bovine IgG-HRP conjugate (Sigma) was used at a concentration of 1/20 000 before the blot was developed using ECL western blotting substrate.

Results

SDS-PAGE & Western Blot

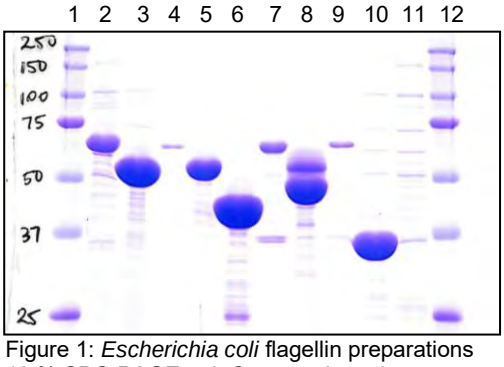


Figure 1: *Escherichia coli* flagellin preparations
10 % SDS-PAGE gel, Coomassie stain

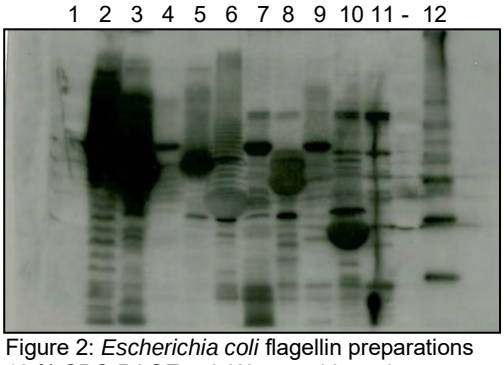


Figure 2: *Escherichia coli* flagellin preparations
10 % SDS-PAGE gel, Western blot using
Travelan batch TRV001 (5 mg/ml)

Lane

1.	Precision Plus marker (Bio-Rad)		
2.	*E. coli* B7A	O148:H28	ETEC
3.	*E. coli* H10407	O78: H11	ETEC
4.	*E. coli* E123-7	O128:H21	ETEC
5.	*E. coli* B2C	O6:H16	ETEC
6.	*E. coli* E11881A	O25:H24	ETEC
7.	*E. coli* LF82	O83:H1	AIEC
8.	*E. coli* K99		Bovine ETEC
9.	*E. coli* E8772/0	O153:H12	ETEC
10.	*E. coli* HS	H4	Human isolate
11.	*E. coli* HB101	non motile	K12 derivative
12.	Fig 1: Precision Plus marker (Bio-Rad) Fig 2: Magic Mark XP (Invitrogen™)		

Conclusions

- The flagellin purification process produced mostly flagella but also had some contaminating proteins and LPS.
- There is antibody to flagellin present in Travelan® which recognises flagellin purified from the motile *E. coli* strains tested.

B. <u>Contribution of flagella-specific antibodies to the efficacy of Travelan against ETEC</u>:

This report presents a study investigating the capacity for flagella-specific antibody contained in Travelan to interrupt two ETEC colonisation strategies that are also important ETEC pathogenic determinants, namely motility and host-cell adherence.

Methods

Purification of IgG from powdered bovine colostrum

Travelan tablets and non-immune powdered colostrum were dissolved overnight at 4^{o}C in PBS (5 ml/tablet and 20 mg/ml, respectively). The suspensions were centrifuged to remove particulate matter and the supernatant adjusted with 2M HCl to pH 4.6 to precipitate the casein component. Following centrifugation to remove the precipitated casein (30 mins, 3200xg, 22^{o}C), the pH of the recovered supernatant was diluted 2-fold in NAb Protein G binding buffer (Pierce, Illinois, USA) and bovine IgG was recovered using the NAb Protein G Spin kit according to the manufacturer's protocol. Purified IgG was further concentrated using Vivaspin 100000 MWCO concentrators. The protein content was estimated by BCA protein assay (Sigma-Aldrich, St Louis, MO, USA) against a bovine serum albumin standard and the [IgG] was adjusted to 5 mg/ml in PBS.

Bacterial strains and culture

ETEC strains H10407 (O78:H11, LT/ST, CFA/I) and B7A (O148:H28, LT/ST, CFA/IV) were cultured for use in assays and flagella purification in 3 ml CFA broth (1% [w/v] casamino acids, 0.15% [w/v] yeast extract, 0.005% [w/v] MgSO4, 0.0005% [w/v] $MnCl_2$) overlaid on CFA agar (CFA broth supplemented with 2.0% [w/v] bacteriological agar). Overlaid plates were cultured overnight at room temperature. The overlay broth was harvested and diluted in PBS to generate a bacterial suspension with an O.D. 600 nm of between 0.3 and 0.4 which equates to approximately 2.5 x 10^8 colony forming units (CFU)/ml. Bacterial suspensions were used neat in motility assays or diluted 1/100 for use in adherence assays.

Purification of flagella from ETEC strains

Overnight cultures (see above) of H10407 and B7A were used to inoculate Luria-Bertani broth. Following incubation (30^{o}C, 80 rpm, 6 hours), the cells were recovered by centrifugation (3200xg, 30 mins, 4^{o}C) and resuspended in 150 mM NaCl/10mM HCl to dissociate flagella from the bacterial cell. Following incubation (1 hour, room temperature, rotation), the cells were removed by centrifugation (8000 x g, 15 mins, 4^{o}C) and the supernatant was neutralised with 50 mM Tris/10 mM NaOH at 12 vol:1 vol. Intact flagella were pelleted from the supernatant by ultracentrifugation (100,000 x g, 90 minutes, 4^{o}C) using a Beckman Optimas TLX with a TLA 100.3 rotor. Pellets were resuspended in PBS overnight at 4^{o}C and contaminating bacterial LPS was removed by two sequential treatments with 12 vols of 0.2% sodium deoxycholate followed by ultracentrifugation, before finally resuspending the purified flagella in PBS.

Caco-2 adherence assays

24-well plates were seeded with Caco-2 cells (ATCC HTB-37) at 10^5 cells/well. The cells were maintained for 20 days (15 days post-confluence), by which time they had fully differentiated to form polarised monolayers with domes mimicking the villus tips of the human small intestine. The culture medium was replaced with 0.25 ml assay medium containing varying amounts of purified IgG and 0.25 ml H10407 (approximately 5 x 10^5 CFU) was added to each well. Following incubation (3 hours, 37oC, 5% CO2:95% air), the monolayers were washed 3 times with PBS to remove non-adherent bacteria, solubilised with 1% Digitonin, and the adherent bacteria were quantified by spreading 10-fold dilutions on Luria-Bertani agar. Samples were assayed in triplicate wells in each experiment, and graphs represent data from 2 independent experiments.

Antigen competition adherence assays were performed by pre-incubating IgG at 0.2 mg/ml in assay media with differing amounts of flagella for 30 mins at room temperature prior to addition to the monolayers.

Soft-agar motility assays

Motility assays were performed in soft CFA agar (CFA broth supplemented with 0.3% [w/v] bacteriological agar). Neat (5 mg/ml) and serial-doubling dilutions in PBS of Travelan or non-immune IgG in 10 µl were stabbed into marked regions of the soft agar and left for 30 mins to absorb into the plates. For antigen competition experiments, one concentration of IgG was pre-incubated with differing amounts of antigen, or varying amounts of IgG were incubated with a single amount of antigen, for 30 mins at room temperature prior to adding to the soft agar. Approximately 5 x 10^5 CFU of ETEC in 2 µl was stabbed into each of the IgG stab sites and the plates were incubated for 8 hours at 37^{o}C. Bacterial motility through the soft agar was visualised using a chemical that turns red in the presence of metabolically active bacteria and the radial diameters were measured hourly from 2 to 8 hours post-inoculation. Duplicate plates were prepared in each assay and the mean diameter was used to calculate the area of motility. Graphs represent the mean of 2 independent experiments.

Conclusions

In these studies, we have shown that IgG purified from Travelan:

1. Significantly reduces adherence of CFA/I producing ETEC strains to a cell-line that mimics the human small intestinal epithelium (Figure 1);

2. Significantly reduces the motility of ETEC strains through soft agar (Figure 2);

3. Binds to both the bacterial surface and flagella (Figure 3); and

4. Has substantially greater reactivity against purified ETEC flagella antigen than IgG purified from non-immune colostrum powder (Figure 4).

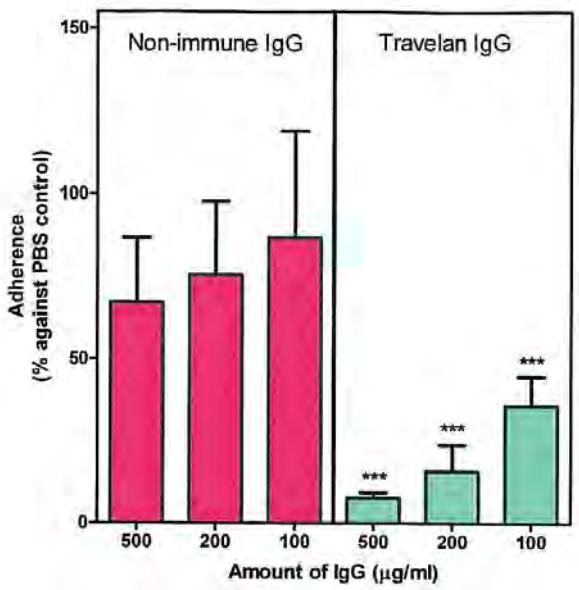


Figure 1 **Antibody-mediated inhibition of ETEC adherence to Caco-2 cells.** IgG purified from Travelan® tablets significantly reduced adherence of ETEC H10407 to Caco-2 cells compared to an equivalent amount of IgG purified from non-immune colostrum in a dose dependent manner (*** P <0.0001, immune versus non-immune at each dose). Bars denote the % adherence compared to PBS controls; mean ± 95% confidence intervals of two independent experiments.

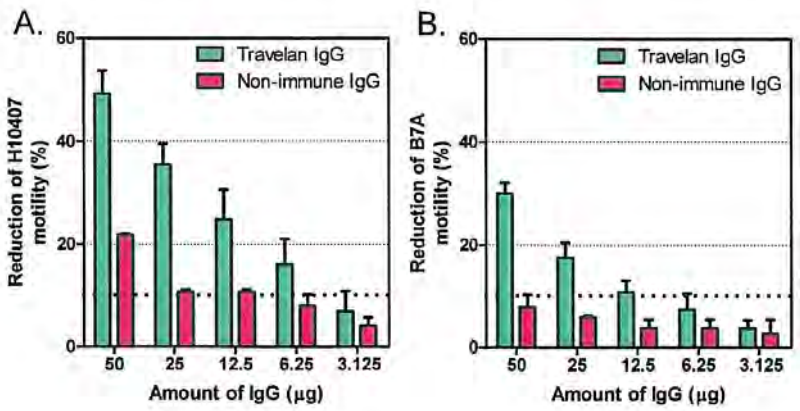


Figure 2 **Travelan® antibody mediates inhibition of ETEC motility.** IgG purified from Travelan® tablets reduced the motility of ETEC strains (A) H10407 and (B) B7A more than that of normal variation in bacterial growth through soft-agar (broken line indicates the limit of normal variation, defined as 1 standard deviation from the mean of the PBS controls included in each assay). NB: IgG purified from non-immune colostrum powder also reduced motility of H10407, but not B7A, at the highest level tested. Bars denote the % reduction in area of bacterial growth through soft-agar compared to PBS-treated control; mean ± standard error of two independent experiments.

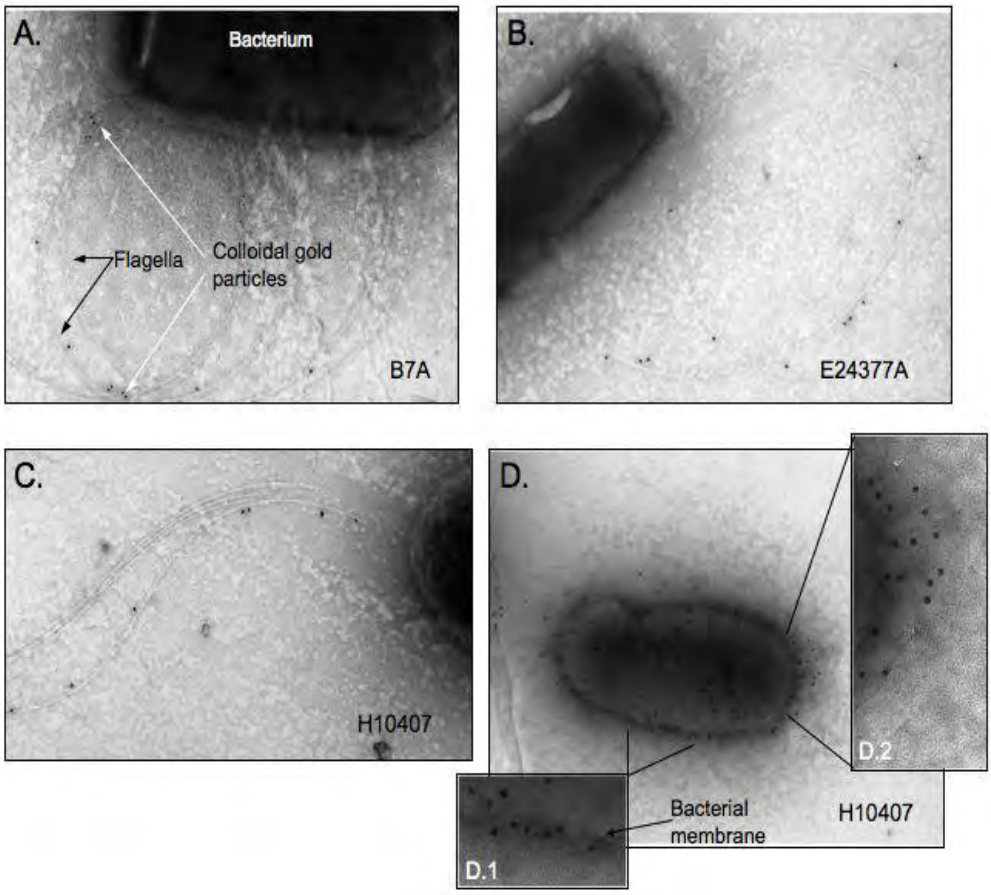


Figure 3 Travelan antibodies bind to ETEC surface-associated structures. ETEC strains cultured overnight at 37oC in CFA broth overlaid on CFA agar were fixed with 1% paraformaldehyde before sequential incubations with Travelan IgG (0.2 mg/ml) and mouse anti-bovine IgG monoclonal antibody (1 in 500, Sigma-Aldrich). The bacterial cells were then adsorbed onto carbon-coated copper electron microscopy grids before a final incubation with colloidal gold-conjugated rabbit anti-mouse IgG (Fc) antibodies (1 in 50, Amersham). The grids were negatively stained with 1% ammonium molybdate, dried and examined by transmission electron microscopy. A) ETEC B7A, gold particles visible on flagella; B) ETEC E24377A, gold particles visible on flagella; C) ETEC H10407, gold particles visible on flagella; D) ETEC H10407, gold particles visible on bacterial cell surface (D.1 shows gold particles localised intimately on the bacterial membrane, suggestive of binding to LPS; D.2 shows gold particles localized distantly from the bacterial membrane, suggestive of binding to fimbrial structures). Bacterium, flagella and colloidal gold particles labeled in panel A indicates features observed in other panels; bacterial membrane labeled in panel D.1 is also visible in panel D.2.

Contribution of flagella-specific antibody to inhibition of ETEC adherence to host-cells:

In order to further investigate the contribution of flagella-specfic IgG to the overall ability of Travelan antibodies to interfere with bacteria/host interactions, bacterial adherence and motility assays were repeated following pre-incubation of the purified Travelan IgG with highly purified flagella recovered from ETEC strains H10407 (O78:H11) and B7A (O148:H28).

When Travelan IgG was pre-incubated with purified H10407 flagella, the ability of the antibodies to inhibit adherence of H10407 to Caco-2 cells was significantly reduced compared to antibody efficacy in the absence of antigen competition (Figure 5). Travelan IgG at 0.2 mg/ml routinely reduced H10407 adherence by 80% compared to non-immune IgG. However, pre-incubation of 0.2 mg/ml Travelan IgG with 0.02 mg/ml H10407 flagella reduced this effect to 50% that observed in the presence of non-immune IgG. This shows

that flagella-specific IgG contributes to the ability of Travelan® antibodies to interrupt interactions between E. coli and the intestinal epithelium.

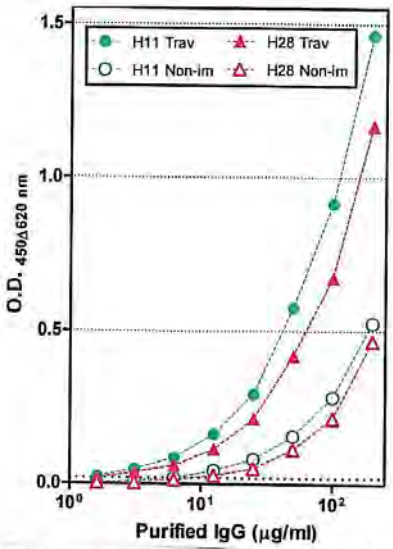


Figure 4 Titration of Travelan® and non-immune IgG against purified ETEC flagella. Non-immune (Non-im) and Travelan® (Trav) IgG was assayed by enzyme-immunoassay against flagella antigen purified from ETEC strains of serotype H11 (open and filled circles, respectively) or H28 (open and filled triangles, respectively). Each IgG dilution was also assayed against an antigen-free well and the resultant background signal was subtracted from that of the test wells to calculate the antigen-specific signal. Each point represents the mean of duplicate wells; the broken line denotes the specific signal cut-off for end-point titration determination. NB. Although non-immune colostrum contains flagella-specific IgG, it is less than one fifth the proportion found in Travelan®.

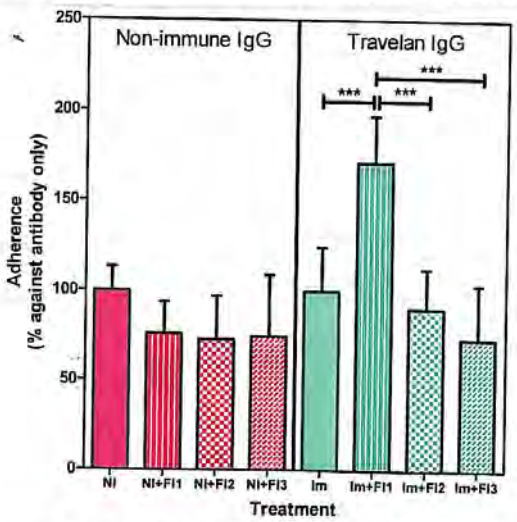


Figure 5 Blockage of flagella-specific antibody can reduce the efficacy of Travelan® antibody-mediated inhibition of Caco-2 cell adherence. IgG (0.2 mg/ml), purified from non-immune (NI) colostrum powder or Travelan® immune (Im) tablets, was pre-incubated with differing amounts of purified flagella (FI1, 20 µg/ml; FI2, 2.0 µg/ml; FI3, 0.2 µg/ml) from H11 serotype strain H10407. Adherence of ETEC H10407 to Caco-2 cells in the presence of pre-incubated IgG is expressed as the percentage of that observed in the presence of NI or Im IgG alone. Bars represent the mean ± 95% confidence interval of triplicate wells from duplicate experiments; *** P <0.0001, 2-way ANOVA with Bonferroni's post-test.

Contribution of flagella-specific antibody to inhibition of ETEC motility:

Pre-incubation of Travelan IgG with purified B7A flagella (H28) completely abolished the ability of the antibodies to inhibit B7A motility compared to antibody efficacy in the absence of antigen competition (Figure 6). However, pre-incubation of the antibody with flagella of a different serotype (H11 from H10407) did not have any negative effect on antibody efficacy. Thus there are various populations of flagella-specific antibodies in Travelan recognising different flagella serotypes, and antibodies that inhibit B7A motility are different to those that bind to H10407 flagella.

These findings suggest that flagella-specific IgG is essential for the ability of Travelan antibodies to diminish E. coli motility and it functions in a serotype-specific manner.

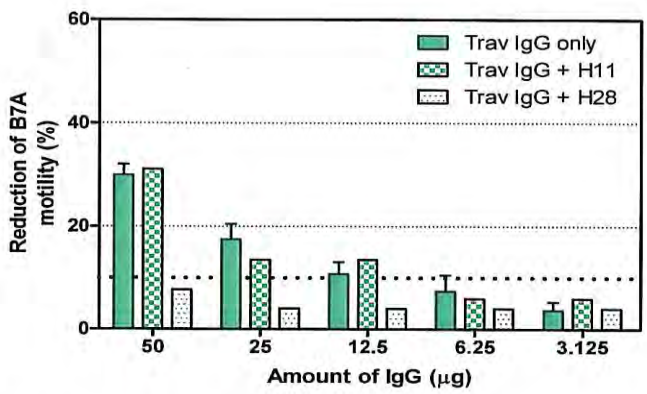


Figure 6 Blockage of flagella-specific antibody prevents Travelan® antibody-mediated inhibition of ETEC motility in a serotype-specific manner. Varying amounts of Travelan® IgG were pre-incubated with 2 μg purified flagella (H11 or H28 from ETEC strains H10407 or B7A, respectively) or PBS, before being used in a motility assay against B7A. Antibody-mediated reduction of B7A motility is expressed as a percentage of B7A motility in the presence of PBS alone; mean ± standard error of two independent experiments. Broken line indicates the limit of normal variation, defined as 1 standard deviation from the mean of the PBS controls included in each assay.

Summary of Results (section 3.6):

Flagella-specific antibodies present in Travelan are able to interrupt two important E. coli pathogenic determinants, namely bacterial motility and adherence to host-cells.

IgG purified from Travelan colostrum:

- Significantly reduces adherence of CFA/I producing ETEC strains to a cell-line that mimics the human small intestinal epithelium.
- Reduces the motility of ETEC strains through soft agar.
- Binds to both the bacterial surface and flagella.
- Has substantially greater reactivity against purified ETEC flagella antigen than IgG purified from non-immune colostrum powder.

3.7 Reduction in ETEC load mediated by Travelan anti-LPS antibody in a mouse respiratory model:

Materials & Methods

Colostrum

- Monovalent Travelan: contained antibodies to H10407 (cows vaccinated with ETEC strain H10407 only)
- Polyvalent -1 Travelan: contained antibodies to 10 ETEC serotypes excluding strain H10407 and including Salmonella

Antibodies

Anti-H10407 and anti- *Salmonella Typhimurium* IgG were affinity purified from monovalent Travelan and polyvalent-2 Travelan respectively. F(ab) fractions were prepared from the affinity purified IgG by papain digestion.

Murine model of ETEC Infection

1. Six week old BALB/c mice were treated with 50 µg in 50 µl of antibody (Travelan IgG, H10407 LPS IgG, F(ab), *Salmonella* LPS IgG and F(ab)) or PBS via intranasal installation, under penthrane anaesthesia.
2. Three hours after administration of antibody or PBS, mice were infected with 2 x 108 CFU of H10407 in 50 µl via intranasal instillation, under penthrane.
3. 48 hours after instillation of H10407 mice were killed and their lungs were removed for bacterial culture.

Results

Murine model of ETEC Infection

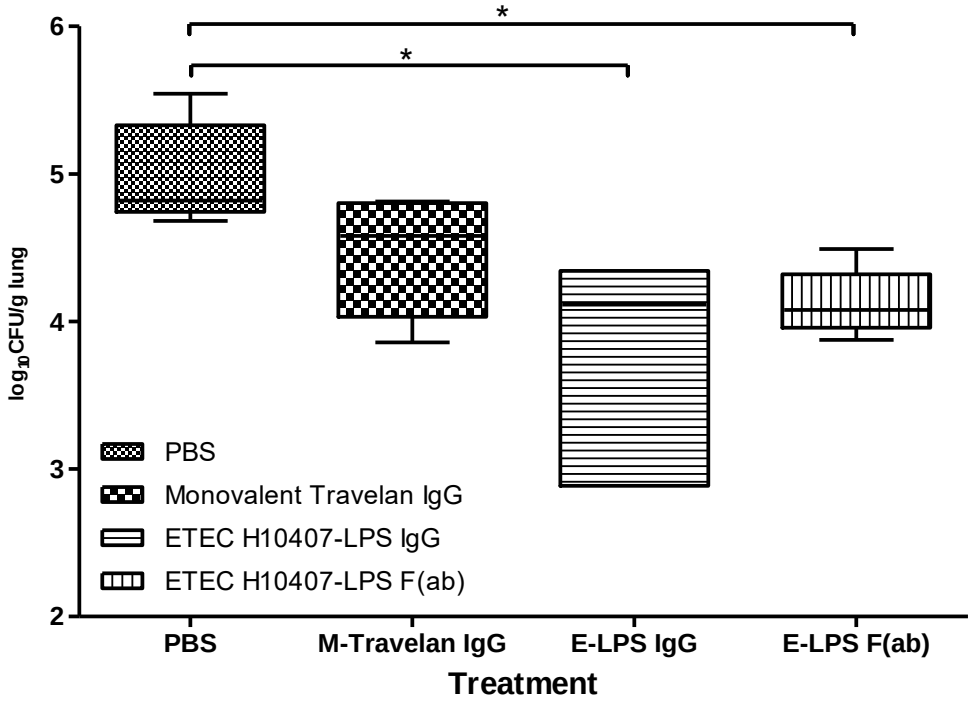


Figure 1: Bacterial load of ETEC H10407 in the lungs of BALB/c mice was significantly reduced when serotype-specific LPS IgG or F(ab) antibodies were delivered before infection (*P<0.05 1-way ANOVA).

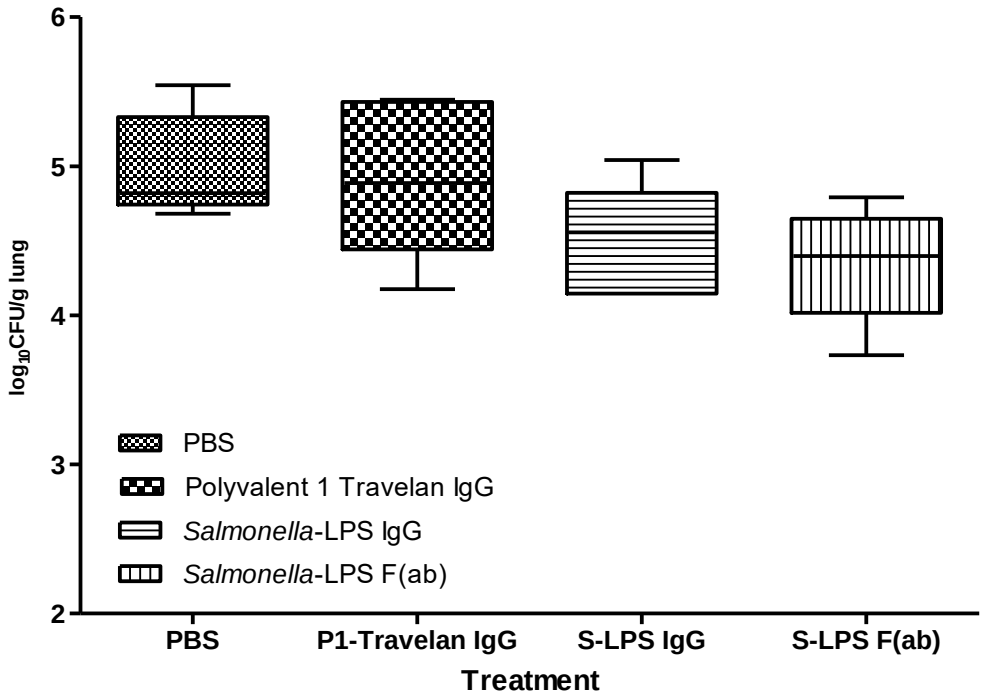


Figure 2: Bacterial load of H10407 in the lungs of BALB/c mice when *Salmonella* LPS antibodies were delivered before infection.

Conclusions

- Mice that received serotype-specific IgG or F(ab)'s showed a significant reduction in bacterial load in the lungs compared to mice that received no antibody.
- Mice given Salmonella LPS F(ab) antibody before infection also showed a reduction in bacterial load compared to control mice, but this reduction was not significant.
- The finding that antibodies to LPS can interfere with epithelial colonisation and infection by ETEC suggests that the anti-LPS antibodies in Travelan contribute to their effectiveness in preventing travellers' diarrhoea.

Summary of Results (section 3.7):

A passive immunity study using a mouse respiratory model showed reduction of ETEC load with homologous and heterologous LPS antibodies:

- It was shown that serotype specific LPS antibody given intranasally prior to ETEC inoculation can reduce bacterial colonisation in the mouse lung.
- Purified CT99 IgG, H10407 LPS IgG, H10407 LPS F(ab) and Salmonella LPS F(ab) fragments from anti-LPS colostrum batch CT99 were all significantly better at reducing infection compared to the PBS control.