Heat-labile enterotoxin enhances small intestinal colonization with F18ac+ verotoxigenic *E. coli* in newly weaned piglets

M. Atef Yekta¹, M. Loos¹, A. Coddens¹, S. Arnouts³, W. Van den Broeck², B. Devriendt, U. Lundberg⁴, D. Epperson⁴, E. Cox¹
Overall hypothesis of the study

LT enterotoxin in subclinical concentrations affects the small intestinal mucosa making it more susceptible to other enteropathogens

- Pig as model
- Enterotoxigenic E. coli as source of LT

If this could be proven vaccination against LT would be a valid strategy

Gregory Glenn
E. coli infections post weaning in piglets

Enterotoxigenic E. coli (ETEC), F4 or F18 fimbriae

Vérotoxinogenic E. coli (VTEC), F18 fimbriae

Fimbriae (F4, F18)

colonisation

Enterotoxins (LT-I, STa, STb)

Diarrhoea

Shiga-toxin (Stx2e)

Oedema disease

Hypothesis: Can an F4 ETEC infection enhance an F18 VTEC infection
Entercyote CFTR = cystic fibrosis transmembrane regulator

PKA = protein kinase A

cGMPKII

Water loss

10^6. coli/g faeces

F4 + ETEC faecal excretion

diarrhoea first week post-weaning

0

2

3

4

5

6

7

Days post infection

2

3

4

5

6

7

Water loss

Thermo-labile enterotoxin (LT) = AB5 toxin

Binds to GM1 Ganglioside on enterocytes

CFTR = cystic fibrosis transmembrane regulator

PKA = protein kinase A

Enterocytne

van den Akker et al, 1996, Structure 4:665-678

Type II

Type I

Enterotoxins (LT-I, STa, STb, EAST1)

F4 fimbriae

MAJOR subunit FaeG = adhesin

Enterotoxins (LT-I, STa, STb, EAST1)
Oedema disease  
second week  
Post-weaning

F18+ VTEC faecal excretion

10⁶ E. coli/g faeces

0 100 200 300 400 500 600 700

2 3 4 5 6 7 8 9 10 11

PMN  
M cell  
Paneth cell  
Sensitive cells  
Apoptosis endothelial cells  

Intestinal epithelium  

Binds to Gb3  

Shiga-toxin (Stx2e)

F18 fimbriae

Minor subunit
FedF = adhesin

F4-specific IgA ASC

F18-specific IgA ASC

Mean ASC per 10^7 cells

Absence of LT

Different interaction of F18 fimbriae?

Verdonck et al., 1999. Vaccine
F4 and F18

Minor subunits

MAJOR subunit FaeG

MINOR subunit FedF

= ADHESIN =

F4 fimbriae

F4ab/ac/ad

F18 fimbriae

F18ab/ac
Binding and uptake of F4

Ligated loops injected with F4

FLUOS-labeled F4

F4 in M cell

Goblet cell

F4 in Enterocytes

F4 in M cell

Cytokeratin-18 18

F4 fimbriae

Snoeck et al., 2008. Vet Immunopath.
Structural insight in binding of blood group A6-1 sugar by the F18 fimbrial adhesin FedF

Moonens et al., Molecular Microbiology, 86, 82-95.
Hypothesis

Can LT enterotoxin in subclinical concentrations affect the small intestinal mucosa of newly weaned piglets so that it enhances F18 VTEC colonization?
F4 + ETEC faecal excretion

Days post infection

F18+ VTEC faecal excretion

Oedema disease
second week
Post-weaning

Verdonck et al., 2002. Vaccine
SMALL INTESTINAL SEGMENT PERFUSION (SISP)

GIS26 (O149:K91:F4ac, LT + STb +Sta+)

F4R⁺ pig 4h perfusion

20 cm segment

GIS26 (O149:K91:F4ac, LT + STb +Sta+)

2.5 x 10⁹ CFU

N = 3 F4R⁺

Absorption

Secretion

F4R⁺ pig 7h perfusion

IMM01 (O149:K91:F4ac, LT + STb⁺)

<table>
<thead>
<tr>
<th>F4⁺ ETEC (Log10)</th>
<th>% Mucus coverage</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>89</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>84</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>73</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>68</td>
<td>8</td>
</tr>
</tbody>
</table>

Net absorption (mg/cm²)

Absorption

Secretion

Loos et al., 2012. PLoS ONE 7(7): e41041; Loos et al., unpublished data
What dose for LT enterotoxin in the SISP of 20 cm?

![Effect of LT on intestinal secretion (average of 3 animals)](image)

After 6 hours perfusion 10 µg was the lowest dose with consistent fluid secretion.

56 ± 70 g fluid per cm² mucosa
What is the effect of these dosages on mucus?

<table>
<thead>
<tr>
<th>LT dose</th>
<th>Number of loops tested</th>
<th>% mucus coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µg</td>
<td>8</td>
<td>89 ± 5%</td>
</tr>
<tr>
<td>3 µg</td>
<td>4</td>
<td>84 ± 4%</td>
</tr>
<tr>
<td>10 µg</td>
<td>3</td>
<td>76 ± 7%</td>
</tr>
<tr>
<td>30 µg</td>
<td>3</td>
<td>60 ± 7%</td>
</tr>
</tbody>
</table>

TEM showing disruption of mucus layer after LT exposure in continuous flow intestinal loop model.
From 20 cm intestinal segments to 8 m intestine in pigs (injection at 5 sites)

<table>
<thead>
<tr>
<th>Pig</th>
<th>BW (kg)</th>
<th>LT (µg)/animal</th>
<th>Euthanasia (hours after LT injection)</th>
<th>Symptoms</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8</td>
<td>250</td>
<td>6</td>
<td>Normal intestinal contents at cecum and rectum</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>500</td>
<td>6</td>
<td>Watery intestinal contents at cecum and rectum</td>
<td>Removal of mucus Normal</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>250</td>
<td>24</td>
<td>Normal feces and some fluid in jejunum</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>350</td>
<td>24</td>
<td>Soft feces and Fluid in ileum and jejunum</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>500</td>
<td>24</td>
<td>Diarrhoea</td>
<td>Removal of mucus</td>
</tr>
</tbody>
</table>

6.25 x 40 = 250 µg

12.5 x 40 = 500 µg
Conclusions

• Intra-intestinal injection of 500 µg LT induces diarrhoea in 4-week-old pigs.

• 250 µg LT did not induce diarrhoea.

• Removal of mucus layer was only clearly detectable in animals which developed diarrhoea.
What is the effect on VTEC colonization

<table>
<thead>
<tr>
<th>LT</th>
<th>F18ab+ VTEC</th>
<th>Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 µg</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>250 µg</td>
<td>10^{11} CFU in 10 ml PBS</td>
<td>2, 3</td>
</tr>
<tr>
<td>350 µg</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>350 µg</td>
<td>10^{11} CFU in 10 ml PBS</td>
<td>5, 6, 7, 11</td>
</tr>
<tr>
<td>0 µg</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>0 µg</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>250 µg</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>250 µg</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>250 µg</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>250 µg</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>250 µg</td>
<td></td>
<td>15</td>
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<tr>
<td>250 µg</td>
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<td>16</td>
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<tr>
<td>250 µg</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>250 µg</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>19</td>
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<tr>
<td>PBS</td>
<td></td>
<td>20</td>
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</tbody>
</table>

6 hours later

<table>
<thead>
<tr>
<th>Pig</th>
<th>250 µg</th>
<th>350 µg</th>
<th>0 µg</th>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>4</td>
<td>8</td>
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<tr>
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<td>3</td>
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<tr>
<td>19</td>
<td>20</td>
<td></td>
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</tr>
</tbody>
</table>

Mean duration
VTEC +LT: 7.0 ± 0.0 days*
VTEC: 5.4 ± 1.4 days

VTEC excretion (F18 Blot)

VTEC colonization of the intestine 3 days after inoculation

VTEC colonization of the intestine 3 days after inoculation
Mucus thin at tips

Pig 19
250 µg LT 6 hours

Pig 7
250 µg LT + VTEC 4 hours

Pig 6
PBS
Conclusions

• Intra-intestinal injection of LT (250 or 350 µg) enhanced colonization with VTEC

• F4+ ETEC producing LT could play a role in colonization of the intestine with VTEC
Acknowledgements

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Valneva Austria
Mechanism of action of LT-I

1. LT (holotoxine) binds to GM1
2. LT is internalized into endosomes
3. LT is transferred to the Golgi apparatus
4. LT is processed and released
5. ATP activates Gsα
6. CL− moves through CFTR

Hirst & D'Souza, 2006, Masignani et al., 2006
<table>
<thead>
<tr>
<th>Toxin</th>
<th>Receptor</th>
<th>Internalization</th>
<th>Intracellular pathway</th>
<th>Target</th>
<th>Cellular alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTI and CT</td>
<td>GM1</td>
<td>Receptor mediated endocytosis</td>
<td>Retrograde transport to Golgi → ER</td>
<td>Protein Gs</td>
<td>ATP → cAMP Adenylate cyclase</td>
</tr>
<tr>
<td>STα</td>
<td>Guanylate cyclase</td>
<td>No</td>
<td>X</td>
<td>Activation of catalytic domain of GC-C</td>
<td>GTP → cGTP Guanylate cyclase</td>
</tr>
<tr>
<td>STβ</td>
<td>Sulfatide</td>
<td>Yes (Receptor mediated endocytosis?)</td>
<td>Unknown</td>
<td>Protein G</td>
<td>↑Ca²⁺ Phospholipases activation</td>
</tr>
</tbody>
</table>

Protein kinase A => phosphorylation

cGMP-dependent protein kinase II => cystic fibrosis transmembrane regulator

Protein kinase C => phosphorylation

**Secretion of**
- Chloride
- Carbonate

**Inhibition of**
- Sodium

**Osmosis-driven water secretion**

**Secretory diarrhea**

Adapted from Dubreuil JD, 2013. Toxins
LT enterotoxin
Sta enterotoxin

Enterocyte

Van den Akker et al., 1996, Structure 4:665-678

Type II

Type I

LT enterotoxin
Sta enterotoxin