Short communication

Effect of plasmid pTENT2 on severity of porcine post-weaning diarrhoea induced by an O149 enterotoxigenic \textit{Escherichia coli}

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Abstract

A particularly virulent O149:H10 enterotoxigenic \textit{Escherichia coli} clone harbours a newly characterized plasmid pTENT2 carrying the tetracycline-resistance \textit{tetA} and the virulence genes \textit{estA}, \textit{paa}, and \textit{sepA} that were not present in less virulent clones. The objectives of this study were to assess whether the additional genes on pTENT2 played a role in the increased severity of post-weaning diarrhoea and if they provided any potential advantage for the emergence of the highly virulent clone. Groups of pigs were dosed orally with isogenic pTENT2-positive and pTENT2-negative ETEC strains, and the clinical and pathological changes were compared between the groups. Two additional groups were given the pTENT2-positive strains and maintained on feed with or without chlortetracycline to assess the effect of subtherapeutic levels of tetracycline on the short-term persistence of the ETEC O149:H10 clone. The severity of diarrhoea within the first few hours post-inoculation was significantly increased \((p = 0.0408)\) in animals receiving pTENT2-positive strains as compared to animals receiving pTENT2-negative strains. There were no consistent or significant histopathological differences between any of the groups and no significant difference in the persistence of ETEC between groups.

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1. Introduction

Enterotoxigenic \textit{Escherichia coli} (ETEC) is a predominant cause of neonatal and post-weaning diarrhoea (PWD) in swine. An increase in incidence and severity of PWD was observed in the swine population in Ontario during the past decade (Josephson et al., 1999). It was associated with the
emergence of a new ETEC O149:H10 clone differing from the O149:H43 ETEC strains previously endemic in Ontario by its resistance to tetracycline and the presence of the estA gene for heat stable enterotoxin STa (Noamani et al., 2003). The estA and tetracycline-resistance gene (tetA) of the O149:H10 ETEC clone are located on a large 85 kbp plasmid (pTENT2) together with the putative virulence genes sepA and paa and two uncharacterized genes named as orfX and orfY (Boerlin and Gyles, 2004). Paa seems to be involved in the development of attaching and effacing lesions (An et al., 1999; Batisson et al., 2003), and sepA mutants of Shigella flexneri are attenuated in virulence in ligated rabbit’s intestinal loops (Benjeloun-Touimi et al., 1995).

Linkages between virulence and antimicrobial-resistance genes have been observed previously (Gyles et al., 1977; Franklin and Mollby, 1983; Martinez et al., 1987; Travis et al., 2006) and raised concerns that use of antimicrobials can lead to the emergence and spread of new pathogens with increased virulence potential (Boerlin et al., 2005). The objectives of this study were to assess: (i) whether the putative virulence genes on pTENT2 were associated with increased severity of diarrhoea, and (ii) whether pTENT2 confers a selective advantage on its host bacterium in the presence and absence of tetracycline in the intestine of pigs.

2. Materials and methods

2.1. Bacterial strains

The ETEC strain AMR029 from a recent PWD case in Ontario (Boerlin et al., 2005) was used as the wild type strain (wETEC). This strain was cured of pTENT2 through electroporation (Heery et al., 1989) followed by screening for loss of tetracycline resistance. The resulting strain (cETEC) was tested by multiplex PCRs for the absence of pTENT2 markers and for persistence of the other virulence genes estB, elt, fae (Bosworth and Casey, 1997) and astA (Ngoleka et al., 2003). The pTENT2 plasmid was reintroduced into cETEC by electroporation using a BioRad electroporation pulser (BioRad, Hercules, California) following standard protocols (Sambrook and Russell, 2001). The restored strain (rETEC) was tested again by multiplex PCRs as described above. The identity of the pTENT2 restriction profiles from wETEC and rETEC were checked using the restriction endonuclease EcoR1 (New England Biolabs, Ipswich, MA, USA). The stability of pTENT2 in rETEC was confirmed by repeated overnight subcultures in LB broth without antimicrobials for approximately 160 generations, while checking the frequency of tetracycline-resistance on LB agar containing 10 μg/ml of tetracycline among 20 colonies recovered from culture on LB agar without antimicrobials at regular intervals. Antimicrobial susceptibility testing using the Canadian Integrated Program for Antimicrobial Resistance Surveillance broth microdilution panel (Government of Canada, 2005) showed identical profiles for all 15 antimicrobial agents tested, except for tetracycline, to which both wETEC and rETEC were resistant and cETEC susceptible.

2.2. Multiplex PCR

A multiplex PCR was developed for detection of pTENT2 using the primer pairs estAp-L and estAp-R, paaAp-L and paaAp-R, tetAp-L and tetAp-R, sepAp-L and sepAp-R, and orfyp-L and orfyp-R (Table 1). The 25 μl reaction mixture consisted of PCR buffer 1 × (Promega, Madison, USA), dNTPs 200 nM each, MgCl2 1.5 mM and 3.75 U of Taq polymerase (Promega). The thermal cycles consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 56 °C for 1 min and elongation at 72 °C for 1 min, followed by a final elongation at 72 °C for 7 min. A second PCR for the tetracycline-resistance genes tetA, tetB, and tetC was developed using a multiplex PCR kit (Qiagen, Hilden, Germany) following the manufacturer’s instruction. The Q-solution (Qiagen) was included in the PCR mix, together with the primer pairs, tetA-R and tetA-L (Lanz et al., 2003), tetBGK-L2 and tetBGK-R2 (Table 1), and tetC-L and tetC-R (Lanz et al., 2003). The thermal cycling was as described above except that annealing was at 63 °C for 1.5 min.

2.3. Animal experiments

The animal experiments were approved by the ethics committee of the Animal Care Services of the
The Yorkshire piglets were obtained from the Arkell Station herd of the University of Guelph and were selected on the basis of homozygote or heterozygote genotypes for markers indicating the presence of F4 specific receptors (Jørgensen et al., 2003 in Rasschaert et al., 2007). The piglets were injected intramuscularly with 150 mg of florfenicol (Schering-Plough Animal Health, NJ, USA) at 20 days of age to eliminate any ETEC colonization or infection before the start of the experiments. The animals were weaned at 21 days of age, and immediately acclimatized in their pens in an isolation unit for 3 days before the start of the experiment. Each experimental group (Table 2) was kept in a separate room and care was taken to avoid cross-contamination between the rooms. Animals from each litter were distributed equally among experimental groups. On day 0, the animals in groups 1–4 were inoculated orally with 109 organisms per animal in 5 ml PBS, 10 min after oral administration of 60 ml of 1.4% NaHCO₃, pH 8.0. Animals in control groups were handled similarly and received 5 ml of PBS without *E. coli*. Chlortetracycline was given at a concentration of 200 ppm to the tetracycline groups (Table 2) in the same standard crumbled starter feed which was fed to all the animals *ad libitum*. Animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Group name</th>
<th>Strain</th>
<th>Tetracycline</th>
<th>pTENT2</th>
<th>Number of animals</th>
<th>Number of animals euthanized</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>W</td>
<td>wETEC</td>
<td>No</td>
<td>Yes</td>
<td>9</td>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>wETEC</td>
<td>Yes</td>
<td>Yes</td>
<td>8</td>
<td>3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>CU</td>
<td>cETEC</td>
<td>No</td>
<td>No</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>RT</td>
<td>rETEC</td>
<td>Yes</td>
<td>Yes</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>None</td>
<td>No</td>
<td>–</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>CT</td>
<td>None</td>
<td>Yes</td>
<td>–</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> These numbers represent totals of three successive replications of the experiment.

<sup>b</sup> One animal had to be euthanized 10 h after inoculation.

<sup>c</sup> Two animals had to be euthanized 10 h after inoculation.

<sup>d</sup> One animal from each group was euthanized on day 11. The post-mortem data from these animals was not used for the study.
were monitored for diarrhoea and rectal temperature three times a day for the first 24 h and daily for the rest of the experiment. The severity of diarrhoea was scored on a scale of 0–4, with 0 being no diarrhoea, 1 for animals with pasty feces, 2 for animals passing liquid feces but with no signs of straining, 3 for the animals passing liquid feces with signs of straining, and 4 for the animals with severe diarrhoea, dehydration and hypothermia. Individual weights were recorded once a day.

Animals were euthanized and post-mortem examinations were conducted on days 1, 7 and 11 post challenge (Table 2). However, the post-mortem data from the animals euthanized on day 11 were not used for the study. Only the clinical data during the first 7 days were used for these animals. Sections of ileum, jejunum and cecum were fixed in 10% neutral-buffered formalin, then sectioned and stained by the Animal Health Laboratory of the University of Guelph. The sections of intestine were assessed qualitatively for villus atrophy, attenuation, adherence of Gram-negative bacilli on the epithelial cells, necrosis of mucosal crypt cells, inflammation within the lamina propria and lympholysis within Peyer’s patches and semi-quantitatively for crypt:villus length ratio. The animals belonging to the same litter were euthanized on the same day across all the experimental groups. Animals manifesting severe clinical symptoms during the study were euthanized prematurely for humane reasons. The histology results obtained with these animals were assigned to the closest group with regard to time of euthanasia.

2.4. Microbiological analysis of intestinal contents

Ten *E. coli* isolates were systematically recovered from the fresh contents of the ileum, cecum and colon of each pig at euthanasia using direct culture on blood agar without antimicrobials. The identification of *E. coli* colonies was confirmed on the basis of lactose fermentation and indole production. All the isolates were stored at −70 °C for further analysis. The isolates from animals euthanized on day 7 were differentiated as ETEC or commensal *E. coli* on the basis of hemolysis on blood agar and multiplex PCR for *estB*, *elt* and *fae* (Bosworth and Casey, 1997). PCR for pTENT2 markers was done on all the haemolytic colonies in groups W, WT, RT and CU. Multiplex PCR for the tetracycline-resistance genes *tetA*, *tetB*, and *tetC* was also done on all the ETEC and commensal *E. coli* isolates collected on day 7.

2.5. Statistical analysis

For the analysis of binary data we used the proc logistic regression in SAS (version 9.1.3, SAS Institute Inc., Cary, NC, USA). We removed the hour of euthanasia and batch (due to handling and space limitations, the experiment was done in three batches) from our analysis because they were non-significant. For non-normal data, we used the Kruskal–Wallis test instead of ANOVA, ignoring the batch effect.

3. Results and discussion

3.1. Disease severity

Except for one animal in group W, all the piglets challenged with an ETEC strain (groups W, WT, CU, and RT from Table 2), developed diarrhoea within 24 h and none of the animals in the control group developed diarrhoea during this period. Differences were observed between the groups (*p* = 0.0408; Fig. 1). In particular, the group (CU) challenged with the pTENT2-negative cETEC had significantly less severe diarrhoea than the groups receiving a pTENT2-positive ETEC (W, WT, and RT in Fig. 1). This indicates that pTENT2 confers increased virulence to ETEC and suggests that at least a part of the increased severity in PWD observed recently in Ontario may be attributable to the presence of pTENT2 (Boerlin et al., 2005). This increased severity of diarrhoea is most likely caused by the *estA* gene carried by pTENT2, but may also be partially enhanced by the two other putative virulence genes *paa* and *sepA*. The individual role of each one of these two genes should now be confirmed using knockout mutants under conditions similar to those of the present study.

The variation in the dynamics of diarrhoea between the three groups challenged with pTENT2-positive isolates is difficult to explain. Part of this may be due to the relatively low number of animals and replicates. Also, the groups receiving the wild type ETEC strain (groups W and WT) suffered a shorter burst of severe
diarrhoea than the group RT. This difference may be an artefact caused by the euthanasia of two piglets in group W and of one piglet in group WT because of very severe diarrhoea 10 h after challenge, thus leaving only animals with less severe signs for the following days (Fig. 1). We can also not exclude the possibility that the slightly delayed development of severe diarrhoea in group RT (Fig. 1) may be an effect of the tetracycline use, confounded by the euthanasia in group WT. Despite the differences in disease severity between pTENT2-positive and -negative ETEC strains, there was no significant difference in the daily weight gained between the different groups until day 7 ($p = 0.9925$).

### 3.2. Histology

Paa and SepA have been shown to increase the adherence of EPEC to intestinal epithelial cells (Batisson et al., 2003) and to cause atrophy of intestinal villi and inflammation of the intestinal mucosa in $S. flexneri$ infections, respectively (Benjelloun-Touimi et al., 1995). In the present study, no significant difference in villus atrophy ($p = 0.8160$), crypt:villus length ratio ($p = 0.4395$), or adherence to intestinal epithelial cells ($p = 0.3840$), was detected between different groups in the animals euthanized on days 1 and 7. There was no clear evidence of inflammation of the intestinal mucosa in any of the groups either. The only statistically significant differences between groups were observed for the attenuation of epithelial cells of the ileum ($p = 0.0273$), an indicator of epithelial cell damage. However, no clear systematic trend was evident with regards to this latter criterion.

### 3.3. ETEC-persistence and tetracycline-resistance genes

To observe whether tetracycline resistance and the pTENT2 plasmid may have provided any advantage for the emergence of the new O149:H10 clone, we assessed the persistence of ETEC in the gut contents of the animals of different groups on day 7. The persistence of ETEC in the animals’ gut was typically high or low (i.e. eight or more isolates out of ten were either ETEC or were non-ETEC). No advantage could be demonstrated for pTENT2-positive ETEC under the selective pressure of tetracycline ($p = 1.0000$), nor in comparison with the pTENT2-negative ETEC when no tetracycline was used ($p = 1.000$). The observation time of seven days may have been too long to observe a disappearance of ETEC (only 2 animals out of 33 were still positive for ETEC on day 7) and the low number of animals per experimental group may also have contributed to this unexpected lack of significant difference. Contrary to what had been observed previously with other resistance plasmids (Poppe et al., 2005), the pTENT2 plasmid was not detected in any of the commensal $E. coli$ isolates recovered on day 7 ($n = 377$). This suggests that, despite the selective pressure of tetracycline in the feed for some groups, pTENT-2 may not be very frequently transferred in vivo between ETEC and commensals. As expected, all the ETEC isolates recovered from the animals challenged with wETEC or rETEC were $tetA$-positive and $tetB$- and $tetC$-negative. Conversely, all the ETEC isolates from the animals challenged with cETEC were negative for all three $tet$ genes. Most of the commensal $E. coli$ carried either $tetA$ ($n = 292$) or $tetB$ ($n = 78$) genes but very few had both ($n = 7$). Unlike in previous field studies (Lee et al., 1993; Maynard et al., 2003), there was no apparent difference in either $tetA$ or $tetB$ distribution in commensal $E. coli$ between any of the
groups \((p = 0.0754\) in ileum, \(p = 0.0930\) in cecum and \(p = 0.8753\) in colon).

In conclusion, our results indicate that pTENT2 is likely responsible for at least a part of the increase in PWD severity caused by the recently emerged O149:H10 ETEC strains. Further studies will now be needed using the parameters set here to investigate whether STa only, or also Paa and SepA are responsible for this difference. Additional points in time other than 24 h post challenge may be needed to demonstrate specific histological changes associated with these virulence factors. Similarly, more intense observation with additional observation time before 7 days may be needed to assess more precisely if the use of tetracycline affects the persistence of pTENT2-positive ETEC.

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References


