Tetracycline use and selection of virulent enterotoxigenic *Escherichia coli*

*Final report*

**Executive summary**

A new strain of enterotoxigenic *E. coli* (ETEC) of the serogroup O149 causing more severe postweaning diarrhea than in the past has emerged during the last decade in Ontario. This strain contains a new plasmid (extra-chromosomal genetic element) called pTENT2, which encodes for tetracycline resistance and for three virulence factors absent from the older O149 ETEC strains previously prevalent in Ontario. This suggests that the increased virulence of the new ETEC strain may be caused by the pTENT2 plasmid and that the use of tetracycline may select for this new strain. The objectives of this project were to develop methods to identify and detect this strain, to generate mutants without the new pTENT2 plasmid, and to compare the behavior of the ETEC strain with and without pTENT2 with and without tetracycline in the feed in an animal model for postweaning diarrhea.

A multiplex PCR was designed and successfully validated for its use to identify *E. coli* strains carrying the pTENT2 plasmid. Unfortunately, after repeated attempts and despite external technical support, we were not able to obtain a satisfactory real-time PCR for the detection and quantification of pTENT2. However, we generated the DNA sequence of the genes necessary for the synthesis of the O149 antigen common to the most prevalent ETEC strains worldwide and in Ontario and designed a real-time quantitative PCR for the detection of *E. coli* O149 in porcine feces. Preliminary results suggest that this PCR is highly specific. Further validation on a larger number of porcine fecal samples will be needed, but this PCR may represent an important tool for diagnostic and epidemiological investigations on post-weaning diarrhea in swine.

Isogenic *E. coli* O149 strains with and without the pTENT2 plasmid were generated. They were used to infect weaned piglets fed with and without tetracycline (200ppm) in the animal isolation unit of the Ontario Veterinary College. The severity of disease and extent of modifications of the intestinal mucosa, as well as behavior of the ETEC strains
and of tetracycline resistance genes were assessed in the piglets. No statistically significant differences were seen for these parameters between groups challenged with the ETEC strain containing or missing pTENT2, or between groups with or without tetracycline. An increase in the severity of the initial diarrhea was seen following the challenge with ETEC in relation with the presence of pTENT2. This change was not statistically significant and would need much larger numbers of animals difficult to manage under experimental conditions to be confirmed. However, these observations fit to the increased severity and more acute nature of post-weaning diarrhea reported by some practitioners and laboratories since the emergence of the new ETEC strain carrying the pTENT2 plasmid.

Introduction

The serogroup O149 is the most prevalent among enterotoxigenic *Escherichia coli* (ETEC) causing neonatal and postweaning diarrhea. Recent studies on ETEC from pigs have shown that a new O149 ETEC variant emerged in the past years in Ontario (Noamani et al., 2003). This new ETEC variant carries a heat stable enterotoxin A gene (*estA*) not found previously in O149 ETEC from pigs in this province and seems to be associated with more severe outbreaks of postweaning diarrhea (Noamani et al., 2003). Antimicrobial resistance and virulence are occasionally linked (Martinez and Baquero, 2002) and increased resistance rates to several antimicrobial agents were observed in isolates belonging to this new ETEC variant (Noamani et al., 2003). In another research project funded by Ontario Pork, we have recently identified numerous associations between antimicrobial resistance and virulence genes in porcine ETEC from Ontario (Boerlin et al., 2005; Travis et al., 2006). Cloning and DNA sequencing work has shown that a tetracycline resistance gene (*tetA*) and the enterotoxin gene *estA* are located on a large transmissible plasmid (called pTENT2 in the rest of this document), together with two additional new virulence gene variants (*sepA* and *paa*) not previously described in ETEC from pigs (Boerlin et al., 2005; Leclerc et al., 2007). These data strongly suggest that the presence of the combined virulence-resistance plasmid pTENT2 is associated with the re-emergence of postweaning diarrhea and increased disease severity in pigs in Ontario. Our recent results show that a large number but not all the recent ETEC isolates from pigs in Ontario carry the pTENT2 plasmid. Some ETEC isolates are similar to older ones and do not have the *estA* gene at all or possess the *estA* gene without the *tetA* resistance gene. Because of the probable clinical significance of pTENT2 and of the diversity observed among ETEC from pigs in Ontario, a rapid PCR test for the differentiation of pTENT2-positive and -negative *E. coli* isolates would be of interest. An additional diagnostic test for the direct detection of *E. coli* O149 and of pTENT2 in fecal samples would also be of great practical interest. The data on the co-location of resistance and virulence genes on pTENT2 strongly suggest that the use of tetracycline in pigs may select for the persistence of pTENT2-carrying ETEC in pigs. Due to the transmissible nature of pTENT2, the use of tetracycline may also possibly increase the spread of
pTENT2 and consequently of the associated virulence genes in other *E. coli* from pigs. However, non-pathogenic tetracycline resistant *E. coli* strains are also widespread in the intestinal flora of swine (Boerlin et al., 2005) and it is not sure how these and the pTENT2-carryers compete under the selective pressure of tetracycline *in vivo*. This needed to be tested before any conclusion could be drawn on the effect of tetracycline use on selection of pTENT2 carriers and on severity of postweaning diarrhea.

**Objectives of the project**

The objectives of the projects were the following:

- Development of a multiplex PCR for the rapid differentiation of pTENT2-positive and negative *E. coli* isolates
- Development of a real time PCR for rapid detection of pTENT2 directly from fecal samples
- Study on the persistence of pTENT2-positive ETEC in pigs and the spread of pTENT2 in *E. coli* populations *in vivo* with and without “in feed” use of tetracycline
- Study of the virulence and persistence of isogenic pTENT2-positive and negative ETEC strains in pigs *in vivo*.

**Work accomplished**

*Multiplex PCR for the identification of E. coli strains carrying the pTENT2 plasmid*

We have shown in previous investigations on the pTENT2 plasmid, that the gene combination of *sepA*, *paa*, *esta*, *tetA*, and a putative gene (*orfY*) is found only in the new ETEC variant carrying pTENT2. A multiplex PCR was therefore developed, which targets all five genes simultaneously.

**Figure 1.** Examples of multiplex PCR results for the identification of pTEN2-positive *E. coli*

Lanes 1, 2, 5, 7, 8, 9, 11, 13, 15, and 16 represent pTEN2-positive ETEC.
Lanes 3, 10, and 14 represent *tetA*-positive *E. coli* without pTENT2.
Lanes 4, 6, and 12 represent pTEN2- and *tetA*-negative *E. coli* strains.
This PCR was validated on a collection of 76 E. coli strains (27 new variant ETEC and 49 other E. coli isolates which included 12 non-pathogenic isolates). The results correlated entirely with the known genotype of these isolates. A few pTENT2-negative isolates showed some PCR products (for instance lanes 4, 6, and 12 in Figure 1), but the size of these products clearly identified them as non-specific and this was not considered a problem.

**Real time PCR for the detection of E. coli strains carrying the pTENT2 plasmid**

Neither estA, nor tetA or sepA alone are specific for the pTENT2-plasmid. However, there are no sequences with significant homology to orfX and orfY from pTENT2 on GenBank and these two sequences are likely specific for pTENT2. Therefore we attempted to develop a real time PCR for the detection of pTENT2 using orfX and tetA as targets. However, despite the involvement of both a private company specialized in primer design for real time PCR (TIB MOLBIOL, Adelphia, NJ, USA) and of the technical support of people from Roche Applied Sciences (Laval, QE), we were not able to develop a satisfactory real time PCR protocol for the desired targets. After a series of unsuccessful experiments (mainly lack of specificity) and on recommendation of the Roche technical support, we had to give up on our intention to develop a real time PCR for the quantification of pTENT2 in porcine feces. Instead, we have concentrated our attention on the detection of E. coli O149. For this purpose, we first sequenced the rfb gene cluster responsible for the synthesis of the O149 antigen (deposited in GenBank under accession number DQ091854). By homology searches on GenBank, we identified two contiguous open reading frames apparently specific for the O149 rfb cluster in E. coli, that we have named orf5 and orf6 (Figure 2).

![Figure 2: The rfb gene cluster of the representative O149 strain (RO8), indicating ORFs, their length in bp, and protein homologies.](image)

A virtually identical rfb cluster has been identified in Shigella boydii (Tao et al., 2005), a bacterium found only in humans and primates but not in swine and other mammals. This is therefore not a source of concern for our objectives, which is use of this sequence for detection of E. coli O149 in swine. Southern blot hybridizations with two probes based
on orf5 and orf6 were performed on a total of 91 E. coli isolates from 64 serogroups other than O149 and all were negative. A sample of 19 recent and old E. coli O149 isolates from Canada and Switzerland was tested similarly and all the isolates were positive. Restriction analysis of long-range PCR products using combinations of the enzymes XhoI, PstI, and HindIII and covering the entire rfb cluster of 38 O149 isolates from Ontario, Quebec, and Switzerland showed identical patterns for all of them. Thus the O149 rfb cluster seems highly conserved across a variety of E. coli O149 strains of different origins. To confirm that no other variable genes outside of the rfb cluster modify the O antigen of the new O149 strains from Ontario, the LPS of a recent O149 representative isolate from Ontario was extracted and purified and sent for determination of the biochemical structure by Evgeny Vinogradov at the Institute for Biological Sciences, National Research Council, in Ottawa. This structure was shown to be identical to the one previously published by others (Adeyeye et al., 1988). Based on these results, we have now designed a real time PCR for the detection and quantification of E. coli O149 in fecal samples targeting the orf5 of the rfb cluster of E. coli O149 (Figure 3). Further validations tests are ongoing with this PCR, but preliminary results using a few porcine fecal samples from farms in Ontario suggest that it is probably specific and sensitive enough for diagnostic application and epidemiological investigations.

**Figure 3.** Example of preliminary results obtained with O149 RT-PCR and porcine fecal samples.

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a) Amplification curve of two positive (NF3A2+VE and POSITIVE) and three negative samples
b) Melting curves of the products from the same samples as in a), showing the specificity of the PCR.

**pTENT2 transfer in vitro and generation of isogenic mutants for animal experiments**

Previous experiments in our laboratory (Boerlin unpublished results) have shown that pTENT2 can be transferred *in vitro* by conjugation between the laboratory *E. coli* strain DH10B and the recipient strain *E. coli* 711Nal. This transfer is of very low efficiency and we investigated if some field isolates of the new O149 ETEC variant would present a better efficiency of transfer. We tested ten different pTENT2-positive O149 wild type isolates in the same way as the laboratory strains and did not obtain any transconjugants. This suggests that the conjugative transfer of pTENT2 between wild type ETEC and other *E. coli* may be regulated and is different from what can be observed between laboratory strains. Thus, transfer efficiency could not be used as a criterion for the choice of ETEC strain for our animal experiments.

A representative ETEC O149 isolate (AMR029) carrying the pTENT2 plasmid (called W in the rest of this report) was selected for the animal experiments and an isogenic mutant cured from pTENT2 (CU) was produced by electroporation. The absence of pTENT2 but lack of other plasmid modifications were confirmed by PCR testing for virulence genes encoded on plasmids other than pTENT2 (*estB, elt, faeG*, and *astA*), and by plasmid fingerprinting. A control strain (R) was generated by reintroducing the pTENT2 plasmid into the cured strain by electroporation. The restored genotype of this strain was controlled by PCR and plasmid fingerprinting in the same way as above and additionally for the five typical genes of pTENT2.

*Animal experiments for the study of pTENT2 and tetracycline use effects*

Most of these animal experiments were setup and performed following the advices and in consultation with C. L. Gyles, Department of Pathobiology, Ontario Veterinary College, and J. Fairbrother, University of Montreal.
Selection of animals for experiments. All the piglets used for the animal experiments were first genotyped for their alleles at the locus of the porcine mucin 4 gene (F4 pilus receptor) following standard protocols (Rasschaert et al., 2007). Only animals with a susceptible genotype were used and animals with a resistant allele were excluded. All the piglets (3 weeks old) were from the Arkell Research Station from the University of Guelph and brought into the OVC isolation unit at weaning, three days before the start of the experiments, for the purpose of acclimatization. Since ETEC are only rarely resistant to this antimicrobial, all the animals were treated intramuscularly with 150 mg of florfenicol 24 hours before leaving the Arkell research station, to avoid as much as possible carry over and contamination of the experimental facility environment by porcine ETEC originating from Arkell.

Preliminary challenge adjustment. In a first step, preliminary experiments were conducted to determine a suitable challenge inoculum with our ETEC strain W. Challenges were conducted with oral inocula of $10^7$, $10^8$, and $10^9$ CFU in a total volume of 5mL PBS approximately 10 minutes after 60mL of a 1.4% sodium bicarbonate at pH 8.0 to neutralize stomach acidity. The animals receiving $10^9$ CFU showed signs of diarrhea within 24 hours but did not need to be euthanized, whereas the animals with a lower inoculum did not consistently show clinical signs of diarrhea. Thus, an inoculum of $10^9$ CFU was considered suitable and used for the rest of the experiments.

Animal experiments. Six different treatment groups were defined for the actual animal experiments using different combinations of challenge strains and tetracycline treatments as described in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Challenge strain</th>
<th>Tetracycline</th>
<th>Presence of pTENT2</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>W</td>
<td>No</td>
<td>Yes</td>
<td>9</td>
</tr>
<tr>
<td>WT</td>
<td>W</td>
<td>Yes</td>
<td>Yes</td>
<td>8</td>
</tr>
<tr>
<td>CU</td>
<td>C</td>
<td>No</td>
<td>No</td>
<td>9</td>
</tr>
<tr>
<td>RT</td>
<td>R</td>
<td>Yes</td>
<td>Yes</td>
<td>7</td>
</tr>
<tr>
<td>C</td>
<td>None</td>
<td>No</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>CT</td>
<td>None</td>
<td>Yes</td>
<td>-</td>
<td>5</td>
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</table>

Chlortetracycline was included in the feed (crumbled starter feed provided ad libitum) at a concentration of 200ppm as indicated in Table 1, starting on the same day as the challenge. These experiments were conducted in three batches, with each batch including animals in every six treatment groups. Except for the two control groups without ETEC challenge (kept in the same room but in separate pens), each group was kept in a separate isolation room. To avoid carry over of virulent ETEC, clothing was changed and boots disinfected between every visit. Every manipulation of pigs was done using fresh gloves.

Animals were surveyed every 4 to 8 hours during the first 24 hours post challenge, then twice a day until end of day 3 and finally once a day until day 7. One to two animals from each group were euthanized and necropsied after 24 hours and the remaining ones at day 7 at which time the experiments were concluded. For humane reasons, animals with signs of severe diarrhea, of severe dehydration, or not able to stand and walk were euthanized and necropsied immediately, at any time during the experiments. Temperature, weight, consistency of feces (using a scale from 0 to 4 with 0 being firm solid feces and 4 watery
feces), and signs of dehydration were registered five, ten, and twenty-four hours after challenge and every 24 hours after that until day 7. Rectal swabs were taken daily for the animals of the two first experimental batches. However, this procedure was stopped after realizing that the microbiological results of these swabs did not correlate with the results obtained on the actual intestinal content of necropsied animals. At necropsy, samples from the content of the ileum, cecum, and colon were aseptically collected. Tissue samples from these three sections of the intestine were also collected for histology on the animals euthanized at 24 hours post challenge (not at day 7, since the animals had practically all recovered at this time). The following histological criteria were evaluated in collaboration with Dr. Josepha DeLay from the Animals Health Laboratory: Attenuation of intestinal epithelium (reduction in length of columnar intestinal epithelium cells: present or absent); Villus atrophy (reduction in length of villi: present or absent); Ratio of crypt length/villus height; Adherence of bacteria to the surface of the epithelium (on an arbitrary scale of 0 to 3). Because of the lack of correlation between rectal swabs and actual content of the intestine, only the intestinal content of the animals sacrificed at day 1 and day 7 were used for the analysis of persistence. For these, ten E. coli colonies were sampled systematically from primary cultures. The number of ETEC colonies and of pTENT2-positive colonies among these 10 colonies were assessed using the multiplex PCR developed in the earlier stages of the project. The number of tetracycline-resistant isolates among the 10 primary colonies and their respective tetracycline resistance genes were assessed by subcultures on tetracycline containing agar and a multiplex PCR developed in our laboratory (Kozak and Boerlin, unpublished), respectively. Statistical analysis was performed in collaboration with William Sears from the Department of Population Medicine at the Ontario College, using multivariable approaches and analyzing the post mortem results and the variables surveyed over the seven days of the experiment separately.

No statistically significant differences were observed between groups for any of the criteria studied. Some trends can be seen in our results, which could possibly be confirmed using larger numbers of animals, unfortunately not manageable under our laboratory and animal isolation unit conditions.

Post mortem histology: On average, the groups challenged with the ETEC carrying the pTENT2 plasmid had more severe attenuation of the intestinal epithelium, but the difference was not significant (p=0.30). With the exception of occasional differences between the control groups not challenged with any ETEC and the ETEC-challenge groups, no clear trend was otherwise visible that would suggest an effect of tetracycline or of pTENT2 on villus atrophy (p=0.82), ratio crypt length/villus length (p=0.44), and adherence of bacteria to the intestinal mucosa (p=0.38).

Disease severity: No significant changes in rectal temperature were observed for the duration of the experiments, except in three animals with such a severe diarrhea that they had to be euthanized and suffered hypothermia in relation with dehydration. No systematic difference was observed between groups with regards to weight gain during the seven days of the experiment (p=0.96). The severity of diarrhea, differed between the groups of animals challenged with ETEC strains, but this difference was again not significant (p=0.12). As illustrated in Figure 4, the animals challenged with the ETEC strains carrying the pTENT2 plasmid (W and WT+RT) seem to have a stronger initial burst of diarrhea than the animals challenged with the ETEC strains devoid of pTENT2.
(CU). This is without taking into account that among the three animals that had to be euthanized on humane grounds between 10 and 12 hours post challenge, 2 were from the W group and one from the WT group. Thus, the real difference between animals challenged with pTENT2-positive ETEC (W and WT+RT), and pTENT-2 negative ETEC (CU) may be stronger than the statistics suggest. There was a noticeable lack of diarrhea at the 48 hours post challenge time point in the animals challenged with the wild type ETEC strain without tetracycline (group W). This may be an artifact related to the loss to the study of two severely diseased animals between 10 and 12 hours post challenge (euthanasia mentioned above). After 7 days, all the surviving animals had practically recovered.

**Figure 4.** Diarrhea scores post ETEC challenge

![Diarrhea scores post ETEC challenge](image_url)

Diarrhea scores ranged from 0 (no diarrhea) to 4 (severe watery diarrhea). CU, animals challenged with cured ETEC strain devoid of pTENT2; W, animals challenged with the original wild type ETEC carrying pTENT2 but not receiving tetracycline; WT+RT, animals receiving tetracycline and challenged with the same wild type strains as W or with the cured strain in which pTENT2 had been reintroduced. Because of the relatively small numbers of animals, the WT and RT groups (both challenged with isogenic ETEC strains carrying the pTENT2 plasmid) were pooled in this analysis to try to increase the power of the statistical analysis. Note that two animals in group W and one animal in group WT were euthanized between 10 and 12 hours post challenge because of severe diarrhea and dehydration.

**ETEC and pTENT2 persistence:** Pure ETEC cultures were recovered from the intestinal content of all the animals challenged with an ETEC strain and euthanized 24 hours post challenge. No difference (p=1.00) was observed after seven days between animals receiving tetracycline and those not receiving tetracycline with regards to the frequency of pTENT2-positive ETEC carriage.

**Tetracycline resistance and tetracycline resistance genes:** No *tetC* gene was observed among tetracycline resistant *E. coli* at day 7. For each individual animal, the overwhelming majority of isolates was either *tetA*-positive or *tetB*-positive and very few
animals carried a mixture of \textit{tetA}- and \textit{tetB}-positive strains. No difference was observed between experimental groups after 7 days with regards to the relative distribution of \textit{tetA} and \textit{tetB}. (p=0.28 to 0.71, depending on intestine section).

\textbf{Conclusions of the animal experiments:} No data were available on the magnitude of p\textsc{TENT2} and tetracycline use effects on disease severity and intestinal or microbiological modifications in postweaning diarrhea. The results of our animal experiments suggest that, contrary to our expectations, these effects are not drastic and our study may lack the power to be able to confirm them. However, our results suggest that the presence of p\textsc{TENT2} may increase the severity of the diarrhea observed during the initial hours of the infection. Unfortunately, because of the individual variation in response to challenge, a much larger number of piglets should be used to confirm these findings. Initial calculations based on our results suggest that more than 60 piglets would be needed to confirm the effect of p\textsc{TENT2} only. These numbers go unfortunately clearly beyond the scope of this project and the logistic limitations of our laboratory.

\textbf{HPQ training}

This project was part of the PhD training of Priti Goswami (DVM) in our laboratory. Thanks to this project, she has been trained in a number of fields and techniques, including classical bacteriology, molecular biology, and animal experiments. She is currently working on preparing two manuscripts with the results of her work in this project. The publications will be sent to Ontario Pork upon publication. This project has also setup the initial steps for the last part of her PhD thesis, which will be on the “on farm” epidemiology of \textit{E. coli} O149.

\textbf{Overall conclusions and outcome}

A new multiplex PCR for the rapid characterization of porcine ETEC and identification of p\textsc{TENT2}-positive strains has been developed in the frame of this project, which will allow to differentiate the two major ETEC O149 variants present in the Ontario porcine population. Technical difficulties have hindered the development of a real time PCR for the quantification of p\textsc{TENT2} in feces and intestinal content of swine. However, our investigations on the \textit{rfb} cluster of \textit{E. coli} O149 and its diversity led to the development of a new quantitative real-time PCR for the detection of this organism directly in fecal porcine feces extracts. Further validation is needed for this PCR, but it may represent an important epidemiological and rapid diagnostic tool in the near future.

The animal experiments conducted in the frame of this project did not allow us to demonstrate statistically significant effects of p\textsc{TENT2} or of the use of tetracycline on clinical and pathological signs associated with post-weaning diarrhea. However, they suggest that the severity of the initial diarrhea observed in our animal model of post-weaning diarrhea could be affected by the presence of p\textsc{TENT2}. This finding is consistent with the more severe and acute nature of post-weaning diarrhea observed when the new p\textsc{TENT2}-positive ETEC strain initially spread in Ontario (DeLay, Archambault, and Gyles, personal communication). Unfortunately, the large individual variability in response to the ETEC challenge, despite our genetic screening for the presence of K88 intestinal receptor, precluded us to provide a formal demonstration of this observation. Much larger numbers of animals would be needed for this purpose. No major effect of
tetracycline use could be observed on the persistence of pTENT2-positive ETEC in the
feces of piglets or on the distribution of tetracycline resistance determinants, respectively.
A large proportion of the normal intestinal flora of swine is resistant to tetracycline and
the tetracycline resistance encoded by pTENT2 may therefore not represent a significant
selective advantage for ETEC in face of these other resistant bacteria under the
tetracycline regimen used in this study. Further studies under more realistic conditions
than those encountered in a close isolation unit may be necessary, but our results suggest
that tetracycline in feed may not have an effect on mid-term persistence of pTENT2-
positive ETEC in the gut of swine.
The experiments performed in this study were new and of exploratory nature. They
probably suffered from a lack of statistical power. However, they provide the basic
numbers needed to design adequate sampling strategies for future comparative field
studies on the virulence of old and new O149 ETEC variants.

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