RESEARCH REPORT
PORK SAFETY

Title: Campylobacter Colonization Dynamids in Pigs – NPB #07-202

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Industry Summary:

Campylobacter are known human pathogens that are capable of causing disease ranging from diarrhea to serious neurological disorders. These bacteria are majorly transmitted to humans via consumption of contaminated foods. Furthermore, *Campylobacter* are highly prevalent in food animals such as chickens and pigs, where they occur without causing obvious clinical symptoms. Accordingly, controlling *Campylobacter* colonization of food animals would limit their transmission to humans, reducing infection episodes. However, due to their fastidious nature, *Campylobacter* are difficult to study using common laboratory procedures, which complicate control efforts. This warrants using new approaches that would allow sensitive monitoring and rapid characterization of these pathogens in their animal host. For this purpose, we generated bioluminescent *Campylobacter* strains, which emit light signals that can be monitored in live animals. This would facilitate colonization studies, clarifying the various stages that are involved in the onset and progression of colonization. We used these strains to study the colonization dynamics of *Campylobacter* in pigs. Our preliminary analysis was promising, showing the colonization of these pathogens in a pig's gut. However, further analyses were complicated by the presence of endogenous *Campylobacter* strains in our test animals that competed with our bioluminescent strains and inhibited their efficiency for colonization. Currently, we are testing different approaches to eliminate indigenous *Campylobacter* from our test pigs. Our preliminary analysis indicated that the bioluminescent strains would constitute powerful tools for studying *Campylobacter* colonization of food animals.

Scientific Abstract:

Campylobacter are Gram-negative bacteria that can cause disease in humans. The pathogens are transmitted primarily through the consumption of contaminated foods. Furthermore, *Campylobacter* is highly prevalent in food animals, where these bacteria occur as commensals. With the absence of vaccine, the reduction of *Campylobacter* in food animals is considered an ideal approach for limiting its transmission to humans. However, control efforts have not been fully successful, partly because the on-farm sources and the colonization cycle of these organisms are not well characterized. Of particular interest is monitoring the colonization dynamics of these pathogens in the animal host, which would elucidate the progression of infection in the affected organs. We hypothesized that constructing bioluminescent *Campylobacter* strains would allow sensitive, rapid, and real-time monitoring of the progression of colonization in pigs. This can be accomplished by using in vivo imaging systems (IVIS) that allow sensitive detection of bioluminescence in animal tissues. Consequently, we constructed two bioluminescent strains of *Campylobacter* (*C. jejuni* and *C. coli*) by transformation with genes that are responsible for light production in naturally luminescent bacteria. These strains were then inoculated into live pigs and infection was monitored for two weeks. Organs from the pigs were harvested throughout the duration of the experiment and visualized using IVIS. Our results did not reveal consistent colonization with the strains and the bioluminescence signals were mostly not detected, even after culturing extracts from the organs onto enrichment media. Further inspection revealed that our test pigs were significantly pre-colonized with *Campylobacter*, which probably competed with the bioluminescent strains, preventing colonization. However, in one experiment, bioluminescence was detected in the intestines, caecum, and mesenchymal tissues of the experimentally infected pigs. The colonization of the latter tissue has not been observed previously. Despite difficulty in acquiring *Campylobacter*-negative pigs, our preliminary results emphasize the potential of bioluminescence in studying *Campylobacter* colonization dynamics in food animals.

Introduction:

Campylobacter species are gram-negative bacteria, which are capable of causing disease in humans (Vandamme and De Ley, 1991). These species are primarily transmitted through consumption of contaminated foods, resulting in diarrhea, abdominal pains, and fever (Black et al., 1988; Jacobs-Reitsma 2000). Furthermore, *Campylobacter* infections result in significant costs associated with medical care, decrease in work efficiency, and on-farm/slaughter management practices. Compounding these problems is the remarkable capacity of *Campylobacter* to survive and persist in the food-animals environments despite being regarded as a fastidious organism under laboratory conditions. It has been shown previously that *Campylobacter* were highly prevalent in pig herds, rendering these animals as a significant source of human infections.

Pigs are natural reservoir of *Campylobacter* species. *C. coli* is the predominant species isolated in pigs (~ 95%), and constitutes; only 11% and 1-6% of the isolates form chicken and cattle, respectively (Nielsen et al 1997). *C. coli* infected pigs can excrete more than 10^7 CFU/g of feces, (Nielsen et al 1997, Alter et al 2005). Though majority of human campylobacteriosis are attributed to *C. jejuni*, campylobacteriosis due to *C. coli* is an economically important health burden resulting in significant number of hospital bed days (Tam et al., 2003). Recent studies have highlighted the importance of *C. coli* infections in humans because of its increased antibiotic resistance to several antimicrobials (Saenz et al 2000, Tam et al 2003). Humans become infected with *Campylobacter* either by direct contact with the feces of infected animals or, cross contamination of pork and pork products. The contribution of porcine *Campylobacters* to human intestinal infections is unclear, and in most countries up to 10 % of human campylobacteriosis is caused by *C. coli*. *C. coli* related human infections are frequent with higher consumption of pork products (Neimann et al., 2003; Studahl A, and Andersson Y. 2002; Malakauskas et al., 2006).

The colonization dynamics of *Campylobacter* in pigs and other animals have not been thoroughly evaluated. This is partly due to the growth properties of *Campylobacter,* which are difficult to isolate and manipulate and are known to enter a physiological state under stress that is not detectable with traditional

laboratory analysis (Gagaiah et al., 2009; Rollins and Colwell 1986). Consequently, novel approaches that provide real-time monitoring of colonization and an assessment of physiological state of the bacteria are needed to study *Campylobacter*.

Bioluminescence has been shown to facilitate real-time monitoring of bacteria in hosts. Auto-bioluminescent bacteria can be genetically engineered by introducing the genes that are responsible for light production in naturally luminescent bacteria (Engebrecht and Silverman 1984). All the genes necessary for light production are carried on the *luxCDABE* operon, (Engebrecht and Silverman 1984; Meighen 1993). This operon was used to engineer different species of auto-bioluminescent bacteria, including important pathogens (Francis et al. 2000). Although manipulation of *Campylobacter* is difficult (Young et al., 2007), the construction of bioluminescent *Campylobacter* species is possible (Kelana and Griffiths 2003).

Here, we describe the construction of 2 bioluminescent *Campylobacter* and demonstrate their use in determining the colonization pattern in pigs.

Objective: To construct bioluminescent *C. coli and C. jejuni* for real-time pathogenesis and persistence studies.

Materials and Methods:

Bacterial cultures and growth conditions:

Selection of strains was based on the strains significance and prior knowledge of its high virulence as well as known to commonly cause clinical infections. One such strain is ST-828, a clone that has been reported in other parts of the world as well as in U.S. causing human infections. This strain has been detected from few pigs in North Carolina and is banked in Gebreyes laboratory. In addition, ATCC and NCTC strains of *C. jejuni* (NCTC11168, ATCC33560) and *C. coli* (ATCC33559) also currently banked in Gebreyes lab, will be used.

Type cultures of *Campylobacter* were grown at 42° C for 24 h under microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2). All enrichments of the *Campylobacter* species were performed using Mueller-Hinton (MH) medium, which was supplemented with the antibiotics when necessary.

Construction of bioluminescent *C. coli* and *C. jejuni*:

The development of bioluminescent *C. coli and C. jejuni* will permit us to better understand the infection process. *Campylobacter* genetics and promoters are poorly studied; therefore, random insertion of the promoter-less *lux* operon will allow isolation of a mutant that constitutively expresses strong bioluminescence using an endogenous promoter. A promoter that is active both *in vitro* and *in vivo* and a bioluminescent mutant that is not compromised in virulence is required for *in vivo* real-time infection studies.

We used several strategies to construct a bioluminescent *C. coli* and *C. jejuni* and the details are described below.

Pig colonization experiments:

Pig experiments were conducted on 3 occasions. Piglets were inoculated orally with the bioluminescent bacteria and monitored for a period of two weeks. Piglets' were sacrificed every 2 days and their organs (intestine, caecum, liver, spleen, kidneys) were harvested and imaged using the IVIS-100 (in vivo imaging system), which detects and quantifies light signals emitted by bacteria in animal tissues. After imaging, the organs were suspended in buffer and macerated to release their contents. The solution was then plated onto enrichment media to detect the bioluminescent bacteria.

Results and Discussion:

***C. jejuni* and *C. coli* strains for the study.**

For the proposed study, we have selected strains based on public health significance, multiple locus sequence typing (MLST) sequence types and multi-drug resistance pattern. We have chosen a highly pathogenic *C. jejuni* strain 81176 that was originally isolated from a patient during an outbreak of campylobacteriosis, this strain exhibits highly invasive in tissue culture cells and shown to be highly pathogenic in monkeys as well as human trials.

We have previously determined the diversity among *C. coli* strains isolated from antibiotic-free and the conventional pig production systems**.** Based on our earlier studies, a *C. coli* strain 4517 (isolated from swine farm in North Carolina) with MLST sequence type 1425 that is resistant to multiple antibiotics is chosen for our studies.

The *lux* genes can be successfully expressed in *C. jejuni* and *C. coli.*

Our successful transformation of *C. jejuni* and *C. coli* with a bioluminescent plasmid suggests the potential of *lux* as a reporter for the study of *Campylobacter* colonization in pigs. We have constructed a bioluminescent plasmid by cloning *luxCDABE* from pXEN-13 into pRY112, a shuttle vector which can be efficiently mobilized from *E. coli* to *C. jejuni and C. coli*. The resulting vector containing the *lux* operon was then successfully introduced into *C. jejuni* and *C coli* by biparental conjugation (**Figure 1**).

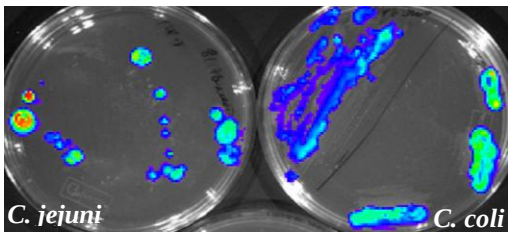

Figure 1. Clones of bioluminescent *C. jejuni and C. coli* generated by introducing modified pRY112/*lux* by biparental conjugation

Construction of a promoter-less *lux::transposon* vector.

To generate stable bioluminescent *C. jejuni* and *C. coli*, we constructed several delivery vectors to integrate *lux* operon in to *C. jejuni* and *C. coli* chromosome.

i) We have constructed a modified EZ::TN transposon vector (pUWGR4) containing a promoterless *lux* operon **(Figure 2).** In pUWGR4, the promoterless *lux* operon was positioned within the transposon mosaic ends such that upon transposon insertion, the expression of *lux* genes will depend on an endogenous *Campylobacter* promoter. This approach involves direct random integration of the *lux* genes into *Campylobacter* chromosome as against the use of bioluminescent plasmids where there is possibility of plasmid loss resulting in under representation of infection. Also, antibiotic pressure needs to be maintained to keep the bioluminescent plasmids stable *in vivo* for the entire duration of study. Therefore, the integration of modified EZ::TN/*lux* transposon by random mutagenesis will allow isolation of a constitutively bioluminescent mutant of *C. jejuni*. In addition, EZ::TN system has been successfully used previously in *C. jejuni* 81176 suggesting the practicality of generating bioluminescent *C. jejuni* using EZ::TN transposon.

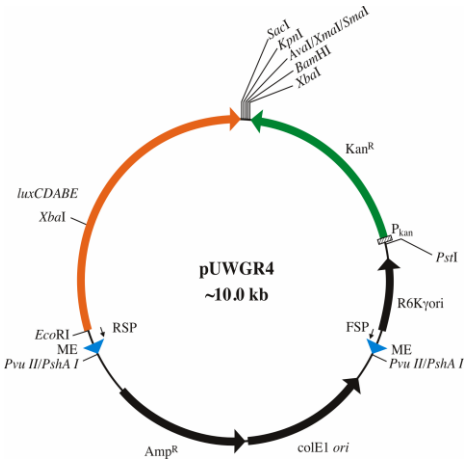

Figure 2. EZ::TN/lux transposon vector for random chromosomal insertion of lux genes into *C. jejuni* and *C. coli.*

ii) Furthermore, we have constructed a suicide vector containing a bioluminescent marker for site directed mutagenesis into *astA* gene of *C. jejuni* 81-176. The *astA* is a non-essential gene in *C. jejuni* 81176 that codes for arylsulfate sulfotransferase which transfers sulfate group from phenolic sulfate esters to a phenolic acceptor substrate. This gene however, is not present in *C. coli* strains. PCR was performed to amplify *astA* gene with 1 kb of flanking DNA sequences from *C. jejuni* genomic DNA. The purified product was then cloned into pZErO-1 plasmid containing the zeocin resistance marker. The *astA* gene was then deleted by performing inverse PCR and replaced with *lux* and kan^R marker from pUC4K (**Figure 3**). The *lux* operon from pXen-13 was then cloned into modified pZERO-1 containing the deleted *astA*.

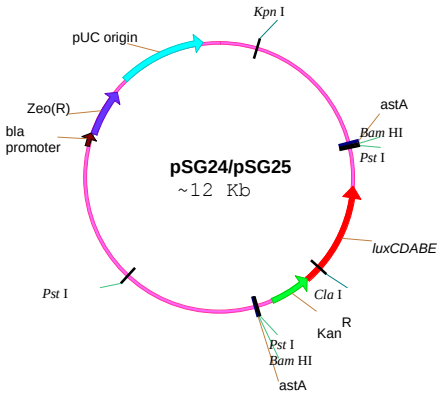

Figure 3. Suicide vector for site directed mutagenesis using *astA*.

iii) Similarly, we have constructed a suicide vector containing a bioluminescent marker for site directed insertion of *lux* genes into *C. coli* 4517 through homologus dependent receptor gene. This gene the *C. jejuni* 81176 genome and our dependent receptor deduced amino genome of *C. coli* RM2228 *C. coli*. Further using primers dependent receptor gene, we have in *C. coli* 4517 and other *C. coli* vector PCR was performed to gene from *C. coli* 4517 genomic then cloned into pZErO-1 resistance marker. A fragment deleted by performing inverse from pXen13 and kan^R marker recombination into putative TonB- was identified as non essential in BLAST analysis of TonB- acid sequence with the unfinished suggested that a homolog exists in specific to *C. coli* putative TonB- confirmed that this gene is present strains. To generate the suicide amplify TonB-dependent receptor DNA. The purified product was plasmid containing the zeocin internal to the gene was then PCR and replaced with *luxCDABE* from pUC4K (**Figure 4**).

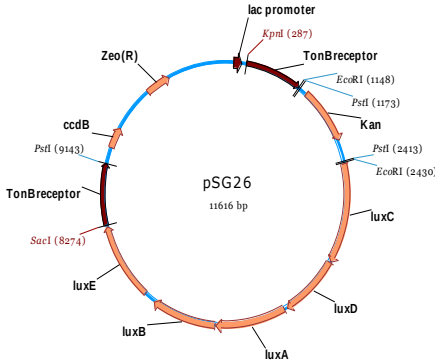

Figure 4. Suicide vector for site directed mutagenesis using putative TonB receptor.

Using the EZ::TN vector or the *astA/tonB* suicide vectors, we were not able to isolate any bioluminescent *C. jejuni* or *C. coli*. Therefore, we constructed another suicide vector based the *C. jejuni* insertional expression vector pRR (provided by Dr. Qijing Zhang, Iowa State University). This vector allows insertion of foreign gene into a rRNA cluster and result in constitutive strong expression of foreign protein. Using this vector, we were able to successfully isolate bioluminescent *C. jejuni* and we are in the processing isolating bioluminescent *C. coli* (Figure 5).

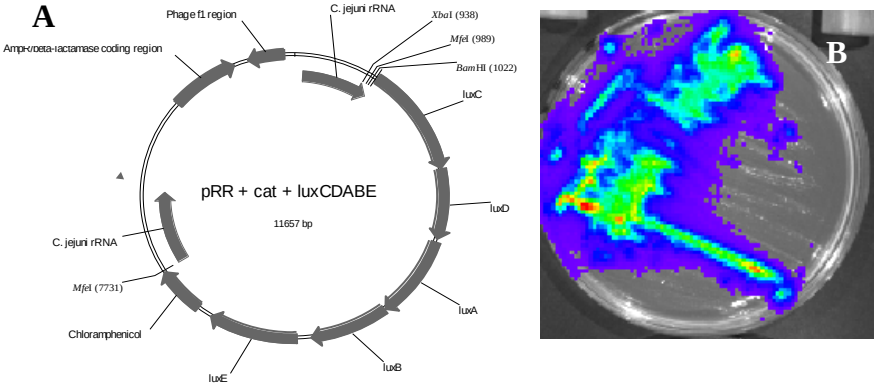

Figure 5. A. pRR/cm/Lux suicide vector used for integrating Lux genes into Campylobacter. **B.** Bioluminescent *C. jejuni* with chromosomally integrated lux into rRNA locus.

Infectivity of *C. jejuni* and *C. coli* strains in piglets.

In order to make sure that the parental *C. jejuni* and *C. coli* strains used for generation of bioluminescent strains colonize piglets. We infected the each parental strain in to 4 piglets that were obtained after they have suckled for 24 hours. These piglets were inoculated with 10^8 bacteria orally and colonization was monitored by determining CFU in feces. Our results indicated that both strains colonize piglet efficiently, however, we found that 50% of our piglets had already colonized with

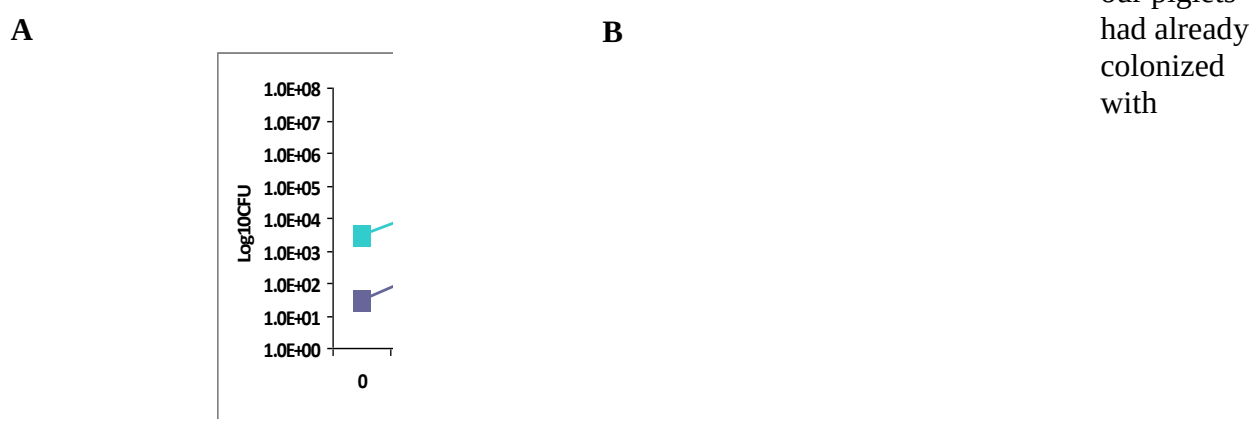

Figure 6. **A**. piglets infected with *C. jejuni*, Cj04 and Cjcontrol (un inoculated) pigs were already precolonized by campylobacter (Day 0). **B.** Piglets infected with *C. coli*, CC02, CC04 and CCcontrol (uninoculated) were already precolonized by *Campylobacter* (Day 0)

Campylobacter **(Figure 6).** Therefore, we decided to amend our protocol and work with pre-suckle piglets.

We have now standardized protocol to raise the pre-suckle piglets using SPFlac, artificial milk product similar to similac. Using the product, we can raise colustrum deprived piglets without mortality.

Pig colonization experiments and future direction:

Our initial experiments with SPF artificial milk were successful. As experimental colonization of the intestines, caecum, and mesenchymal tissues were observed as early as 1 week after inoculation (data not shown). However, these were only conducted on a limited number of Gnotobiotic piglets (n=2). Unfortunately, the experiments that followed in conventional piglets (n=20) were complicated by the occurrence of pre-colonization of these piglets with *Campylobacter*. Despite our efforts to amend our protocols, limiting contamination, using sterile milk and maintaining good sanitation and even proceeded to catch the piglets directly at delivery from the sows and transfer them to containment facilities without exposing them to environment or sows, pre-colonization persisted and we believe that it affected the experimental challenge with the bioluminescent strains, possibly through competitive exclusion. Consequently, consistent conclusions could not be drawn from our trials.

To solve these problems, we are amending our experimental protocols to include antibiotic treatment of the piglets for 2 days, followed by 2 days of no antibiotics and then challenging with the bioluminescent strains. During the experiment, piglets will be maintained in isolation bubbles and provided with sterilized feed. Further, we will increase the frequency and concentration of the challenge inoculums. We also will attempt to introduce sturdier bioluminescent species such as *E. coli* to be used as control. Since we have established a successful protocol to generate bioluminescent *Campylobacter* and since *C. jejuni* that was used in this study is from a human source, we will construct bioluminescence in *C. jejuni* and *C. coli* strains isolated from pigs. We believe that these strains would be more suitable to compete with indigenous bacteria in the pig's gut, facilitating its colonization. These approaches will provide better chances for successful colonization with the bioluminescent strains.

Acknowledgements:

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