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To the Graduate Council:

I am submitting herewith a thesis written by Yingying Hong entitled "Evaluation of chromosomally-integrated luxCDABE and plasmid-borne GFP markers for the study of localization and shedding of STEC O91:H21 in calves." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Alan G. Mathew, Major Professor

We have read this thesis and recommend its acceptance:

Jun Lin, David A. Bemis, Arnold Saxton

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Yingying Hong
May 2011

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ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to everyone contributed to the completion of my Master's project.

Especially, I wish to express the most thanks to Dr. Alan Mathew, my major advisor, for his detailed guidance, constructive suggestions and continuous support throughout my Master's study and for offering me the opportunity to continue the Ph. D. study in Purdue University. Likewise, I would like to thank Dr. Jun Lin, Dr. David Bemis, and Dr. Arnold Saxton for serving on my committee. In particular, I would like to thank Dr. Lin for sharing his laboratory resources. It has been a delightful experience to work with his wonderful team.

I also would like to thank all the colleagues in our laboratory, Drs. Ximin Zeng, Ky Hoang Van, Fuzhou Xu, Ms. Andree Hunkapiller and Mr. Wei Zhou, for their patient help in every aspect of my work in the laboratory. Special thanks to Dr. Fernando Alfonso Di Croce for his experienced assistance in my animal experiment, and to Mr. Jason Shultz, Ms. Heather Anne Bayko, Ms. Portia Greenlee, and all JARTU staff for the excellent maintenance of my calves. Thank you to Dr. Nora Lia Padola for providing the *E. coli* strains and Dr. Steven Ripp for access to IVIS system.

Last, but most importantly, I would like to thank my parents for their everlasting love, support and understanding.

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) has been recognized as an important foodborne pathogen. Of this group, O91 is one of the common serogroups frequently isolated from patients and food in some countries, with O91:H21 being previously implicated in hemolytic uremic syndrome (HUS). Cattle are principle reservoirs for STEC, and studies examining STEC shedding in cattle often include experimental inoculation of strains of interest using antibiotic resistance markers for identifiable recovery. However, indigenous fecal microbes exhibiting similar resistance patterns can confound such studies. Such was the case in a study by our group when attempting to characterize shedding patterns of O91:H21 in calves, leading us to seek other, more effective, markers. Among our strategies was the development of a chromosomally integrated bioluminescence marker via transposon mutagenesis using a *luxCDABE* cassette from *Photorhabdus luminescens* and a plasmid borne GFP marker via transformation of the pGFP vector. The *luxCDABE* marker was inserted on host chromosome at a site that was 27 nucleotides before the stop codon of gene *yihL* and confirmed to have little impact on important virulence genes and growth rate with a very high stability. In contrast, plasmid borne GFP marker showed poor stability without the application of appropriate antibiotic selection pressure. For calves receiving *luxCDABE*-marked O91:H21, the fecal counts of the organism ranged from 1.2 x 10^3 to 1.3 x 10^4 CFU/g at two days post inoculation and

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decreased to 5.8 to 8.7 x 10^2 CFU/g or undetectable level after two weeks. Intestinal contents sampled from various positions at day 14 post inoculation indicated that cecum and descending colon may be the primary localization sites of this O91:H21 strain. Compared to antibiotic resistance markers, the use of bioluminescence markers does not require the restricted pre-inoculation screening of animals. The enumeration of *luxCDABE*-marked O91:H21 from feces and intestinal contents was easily accomplished and confirmed reliable by M-PCR analysis under the presence of indigenous bacteria which cannot be eliminated by antibiotic-supplemented selective plates. Therefore, the chromosomal integrated *luxCDABE* marker may be a better model for the study of STEC colonization and shedding in cattle.

Key Words: shiga toxin-producing *Escherichia coli* (STEC), shedding, *luxCDABE*, GFP, marker, O91:H21

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LITERATURE REVIEW

Introduction

During the past two decades, shiga toxin-producing *Escherichia coli* (STEC) has emerged as one of the top foodborne pathogens of concern. According to the last estimate in 1999, STEC causes approximately 100,000 illnesses every year in the US, resulting in 3,000 hospitalizations and 90 deaths (Mead, Slutsker et al. 1999). Diarrhea is the most common clinical manifestation among patients (Coia 1998) and is frequently accompanied by blood in the feces. Some patients, particularly those at the extremes of age, may further develop life-threatening complications such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Griffin and Tauxe 1991; Paton and Paton 1998; Blanco, Blanco et al. 2004).

Ruminants, especially cattle, are considered the principal reservoir for STEC. Under natural exposure, infection in cattle only leads to asymptomatic carriage, with the organism confined within the intestine and shed via feces. Cattle can persistently shed the organisms in their feces from a few days to several months (Shere, Bartlett et al. 1998; Menrath, Wieler et al. 2010). Under typical environmental temperatures, some STEC can survive in feces for more than 49 days (Wang, Zhao et al. 1996). Contaminated feces can lead to cross-infection or reinfection on the farm, contamination of equipment and meat products during processing, and direct or indirect contamination of fruits and vegetables when manure is used for fertilization or contaminates irrigation water.

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A clear understanding of various STEC shedding patterns in cattle and factors that could affect the duration and magnitude of the shedding might help limit STEC carriage in animals and contamination in food. In common practice, studies examining STEC shedding in cattle usually include experimental inoculation of strains of interest using antibiotic resistance markers for identifiable recovery. However, such studies can be confounded by similar antimicrobial resistance patterns exhibited by indigenous microflora. To date, approximately 40% of manufactured antibiotics have been used in livestock production systems, especially as feed supplements for growth promotion purposes (Levy 1998). Frequent exposure of farm animals to antibiotics creates selection pressure that may result in an increased proportion of antimicrobial resistant bacteria in an animal's fecal flora.

To overcome such problems and to satisfy long-term research strategies of STEC colonization and shedding in cattle, other more effective markers are urgently needed. Among various options, bioluminescent and fluorescent markers appear to be good candidates and their non-destructive physiological characteristics facilitate real-time monitoring, which could be valuable for *in vivo* studies in the future. Bioluminescent markers were of particular interest in the current study because they are easily detected and have less or no background interference from host enzymes or culture media.

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STEC classification

The term STEC refers to the ability of the organism to produce a toxin (Stx1, Stx2, and Stx2 variants) that is homologous to the shiga toxin type I of *Shigella dysenteriae* (O'Brien, LaVeck et al. 1982). STEC is also commonly referred to as verocytotoxin-producing *Escherichia coli* (VTEC) based on the observation that shiga toxin had direct cytotoxic effects on cultured Vero cells (African green monkey kidney cells) (Konowalchuk, Speirs et al. 1977).

Currently, more than four hundred STEC serotypes (Blanco, Padola et al. 2004) have been identified based on the recognition of their O (somatic lipopolysaccharide) and H (flagella) antigens (Nataro and Kaper 1998).

Among STEC, O157:H7 is the most notable serotype. It was first isolated from a California patient with bloody diarrhea in 1975. However the public health significance of this organism was not of high concern until it caused a multistate outbreak of hemorrhagic colitis (HC) associated with a fast-food chain in 1982 (Riley, Remis et al. 1983). It is estimated that O157:H7 causes approximately 73,000 infections and 60 deaths every year in the US, representing two thirds of total STEC infections (Tserenpuntsag, Chang et al. 2005). HUS cases caused by the organism account for more than 80% of typical HUS cases in North America, where HUS is the leading cause of acute renal failure in children (Banatvala, Griffin et al. 2001). The delayed or defective sorbitol fermentation feature in O157:H7 distinguishes it from other *E. coli* strains and has provided a convenient detection method for routine screening. Since 1996, O157:H7 infections have

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been monitored under the Foodborne Diseases Active Surveillance Network (FoodNet), a component of the CDC's Emerging Infections Program (EIP), in collaboration with the USDA and FDA. According to their most recent report, a significant decrease was observed in the incidence of reported O157:H7 infections in 2009, compared to that in the previous 3 years (CDC 2010).

Food, especially beef products, is considered the major source of O157 infections. Historically, several large outbreaks were associated with consumption of contaminated ground beef, including incidences in 1982 and 1993 (Rangel, Sparling et al. 2005). Therefore, in 1994, the USDA Food Safety and Inspection Service (FSIS) declared O157:H7 as an adulterant in food, which allows agents to seek a recall if food is confirmed to be contaminated with O157:H7.

Compared to infections caused by other STEC serotypes, O157:H7 infections appear to be more likely to result in severe illnesses. In the US, approximately 80% of diarrhea patients infected with O157:H7 develop HC, compared to only about 40% in non-O157 STEC infections; meanwhile, 8% of HC caused by O157:H7 may progress to HUS, whereas HUS is rare in non-O157 STEC infections (CDC, 2007). In some other countries, where O157:H7 is overall less prevalent, such as Argentina which holds the world's highest incidence of HUS in children, O157:H7 was involved in 50-60% of HUS cases (Bustamante, Lucchesi et al. 2009). In Europe, several extensively-conducted surveys concluded that although 80% of STEC isolates from patients with diarrhea

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belonged to non-O157 serogroups, approximately 81% of isolates from HUS patients were O157:H7 (Parma, Sanz et al. 2000).

Infections caused by non-O157 STEC were documented almost the same time as those of O157:H7 (Karmali, Petric et al. 1985). However, the infection incidence is typically underestimated partly due to the challenges of developing rapid identification techniques. Meanwhile, since most clinical laboratories don't routinely screen for these organisms in diarrhea stool samples, only isolates associated with outbreaks or severe illnesses have been reported. In the US, surveillance data associated with non-O157 infections in FoodNet has only become available since 2000. The number of reported infections has increased every year since then. In 2000, non-O157 infections only represented 5.5% of the STEC infections, whereas in 2009, the percentage increased to 36.5% (CDC 2010). In other countries, the frequency of non-O157 infections among total STEC infections varies, ranging from 19 to 100% (Johnson, Thorpe et al. 2006).

Over one hundred non-O157 STEC serotypes have been associated with human diseases. However, these serotypes are not equally pathogenic. Some serotypes are capable of causing illnesses indistinguishable from those caused by O157:H7, while some others are only capable of causing diarrhea. Pathogenesis of each serotype largely depends on its virulence profile. However, genomic analyses of O157, O26, O111 and O103 found a surprisingly great inclusion of mobile genetic elements including prophages, integrative elements and plasmids, with many virulence-conferring genes located in these regions

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(Ogura, Ooka et al. 2009), which may contribute to an increased virulence in previously low-virulent strains, via horizontal gene transfer.

Predominant non-O157 STEC in outbreaks is limited to only several serogroups and may vary geographically. In the US, infections caused by six serogroups, including O26, O103, O111, O121, O45 and O145, represent more than 70% of total non-O157 infections (CDC 2010). Due to several recent outbreaks related to O145 and O26, there has been pressure from the public to broaden the adulterant criteria to at least these six most frequently isolated non-O157 STEC serogroups. However, opposing voices from The American Meat Institute stated that non-O157:H7 STEC has not yet become a public health emergency in beef products. In Europe, O26, O111, O145, O103 and O91 are the five most common serogroups among isolates from patients. Serogroup O91, although rarely reported in the US, is the most frequent serogroup among adult STEC infected patients in Germany (Werber, Beutin et al. 2008).

Putative virulence factors

Shiga toxins (Stxs) are directly associated with the development of HC, HUS and TTP complications. They can be divided into two major groups, Stx1 and Stx2, based on the similarity level of amino acid sequence to Stx of *Shigella dysenteriae*. Compared to Stx1, Stx2 has only approximately 50-60% aa-homology to the Stx of *S. dysenteriae* (Weinstein, Jackson et al. 1988) and cannot be neutralized by anti Stx of *S. dysenteriae* and anti Stx1 immunoglobulins. Moreover, Stx2 possesses more heterogeneity, with variants

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Stx2, Stx2c, Stx2c2, Stx2d, Stx2e and Stx2f being recognized in different STEC serotypes (Muthing, Schweppe et al. 2009). Generally, bacteria able to produce Stx2 are considered more virulent than those that only produce Stx1.

Stxs are comprised of a catalytic A subunit of 32-kDa and a pentameric B subunit (7.7-kDa for each monomer) that binds the toxin to specific cell surface receptors (Paton and Paton 1998). Most Stx variants bind specifically to Gb_3 (globotriaosylceramide) receptors (Lingwood, Law et al. 1987) with the exception of Stx2e which preferentially binds to Gb4 (globotetraosylceramide) (DeGrandis, Law et al. 1989) receptors. The human kidney expresses high levels of Gb_3 in both the cortex and medulla (Boyd and Lingwood 1989), which may explain the fact that the kidney is one of the most affected organs in severe STEC illnesses. On the other hand, human large intestinal epithelium, which is considered the major site for STEC colonization (Paton and Paton 1998), lacks the presence of Gb3 receptors (Holgersson, Jovall et al. 1991), and therefore is not affected by the cytotoxicity of Stxs. Various studies have showed the translocation of both Stx1 and Stx2 from apical to the baselateral side of the Gb_3-negative epithelial cell monolayer, without observing apparent cell damage (Acheson, Moore et al. 1996; Schuller, Frankel et al. 2004). It is currently proposed that transcellular transcytosis, in particular through the macropinocytosis pathway (Maluykova, Gutsal et al. 2008), may facilitate translocation of Stxs across the intestinal epithelium to underlying tissues and into the blood circulation causing damage to the endothelium, which may also induce mucosal damage via ischemia or

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apoptosis signals (Schuller, Frankel et al. 2004) and create the translocation pathway through epithelial lesions.

Upon binding to the receptor, Stxs are internalized and undergo retrograde transport via the Golgi to either the endoplasmic reticulum (ER)/nucleus that can lead to high cytotoxicity (Arab and Lingwood 1998) or to endosomal/lysosomal compartments that leads to toxin inactivation, as has been observed in bovine tissues (Hoey, Sharp et al. 2003). During internalization, the C-terminal of the A subunit is cleaved to generate the form with ribosomal RNA N-glycosidase activity with the potential to inhibit protein synthesis through cleavage of an adenine residue from the 28s rRNA subunit at the position where elongation factor 1-dependent binding of aminoacyl tRNA to the 60s rRNA takes place (Saxena, O'Brien et al. 1989), and further leads to cell apoptosis.

Pathogenicity is also facilitated by the ability of the organism to adhere to the intestinal mucosa and colonize in the gut. The *eae* gene has been widely used as an indicatior of potential efficient colonization. Its product, an outer mambrane protein, called intimin, mediates an intimate attachment of STEC to the surface of enterocytes. This process, together with the effacement of brush border microvilli and cytoskeletal rearrangement, is defined as attaching and effacing (A/E) adherence (Shaw, Cleary et al. 2005), which is mediated by a chromosomally-located pathogenicity island named the locus of enterocyte effacement (LEE). Besides intimin, LEE also includes genes coding a translocated intimin receptor (Tir), a type III secretion system (TTSS) and some secreted effector proteins such as EspA, EspB and EspD, which are considered

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to be involved in the initial host-bacterium interaction and Tir translocation (Ebel, Podzadel et al. 1998). LEE-positive STEC includes several serogroups that are commonly implicated in HUS cases and outbreaks, such as O157, O26, O103 and O145 (Padola, Sanz et al. 2004).

In certain LEE-negative STEC, such as O113:H21 and O91:H21, an alternative adhesin, STEC autoagglutinating adhesin (Saa) has been identified (Paton, Srimanote et al. 2001). Saa is encoded by single open reading frame located on a virulent megaplasmid. STEC naturally carrying the *saa* gene, as well as *saa*-transformed *E. coli* K12 strain, showed high adherence to epithelial cells with phenotype different from that of typical LEE-induced A/E adherence. Only microvillus effacement, but neither cytoskeletal rearrangements nor intimate adherence, was observed (Dytoc, Ismaili et al. 1994; Paton, Srimanote et al. 2001). It is proposed that the number of a 37-aa repeated element in the C-terminal of Saa may affect adherence properties (Paton, Srimanote et al. 2001). Beyond the above, other mechanisms or factors affecting Saa-mediated adherence are not clear.

In addition to intimin and Saa, other adhesins have been recognized from both LEE-positive and negative STEC. The chromosomally encoded adhesin, Iha, is one example (Tarr, Bilge et al. 2000; Newton, Sloan et al. 2009).

The harboring of other accessory virulence factors could enhance STEC virulence. Enterohemolysin (EhxA) is found in most of the STEC isolates associated with human diseases. This plasmid-encoded hemolysin showed

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cytotoxicity to many cell lines and may function synergistically with Stxs (Schmidt, Beutin et al. 1995). By lysing erythrocytes, hemoglobin is released as a potential source of iron (Law and Kelly 1995), influencing the survival of STEC in the iron-limiting intestinal environment. The *rpoS* gene was reported to mediate an acid tolerance response upon the exposure of STEC to stomach acid (Gorden and Small 1993). The SubAB toxin could contribute to severe illnesses through single-site cleavage of an ER chaperon (Paton, Beddoe et al. 2006) and is more prevalent in LEE-negative serotypes (Newton, Sloan et al. 2009).

STEC infections in human

STEC infections in humans can lead to a spectrum of diseases, ranging from asymptomatic carriage and watery diarrhea to life-threatening complications, such as HUS and TTP (Griffin and Tauxe 1991; Paton and Paton 1998; Blanco, Blanco et al. 2004). Diarrhea typically starts 3-4 days post-ingestion. In approximately 40-80% of cases, diarrhea progresses to HC, which is characterized as visible blood in feces and, frequently, severe abdominal cramps and vomiting (Riley, Remis et al. 1983). Most patients recover from HC within approximately one week. However, for a small percentage of patients, especially children less than 4 years old and the elderly and immune-compromised patients, HC can be life-threatening.

Approximately 7% of HC cases in O157:H7 infection progress to HUS (Coia 1998). The rate is not well investigated for infections caused by the various non-O157 serotypes, but is considered to be lower. Patients usually develop

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HUS approximately one week following the onset of diarrhea. The clinical features include microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure (Coia 1998). Syndromes may be further complicated by the involvement of damage to the central nervous system (CNS) and other organs. HUS has a mortality rate of 3-5%, and as high as 30% of HUS patients suffer permanent complications including chronic renal failure and hypertension (Tarr 1995).

TTP is another complication more commonly observed among adult patients. Clinical features are similar to that of HUS, except renal damage is relatively less pronounced in TTP; instead, patients tend to suffer more neurological impairments, including severe headache, stroke, seizure, and CNS deterioration (Morrison, Tyrrell et al. 1986).

Because diarrhea, abdominal cramping and bloody stools are not specific to STEC infections, the course of diagnosis is frequently prolonged and non-specific therapies are applied to patients. In the case of HUS, hemodialysis and blood transfusion are often required. Antibiotic treatment is controversial in STEC infections. Certain antibiotics functioning as DNA synthesis inhibitors, such as mitomycin and ciprofloxacin, were showed to induce Stx-encoding bacteriophage and increase Stx production (al-Jumaili, Burke et al. 1992; Walterspiel, Ashkenazi et al. 1992; Matsushiro, Sato et al. 1999; Zhang, McDaniel et al. 2000), which could increase the risk of HUS development. Although such effects have not been observed with antibiotics that target cell wall synthesis, transcription

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and translation (Kurioka, Yunou et al. 1999; McGannon, Fuller et al. 2010), antibiotic treatment is currently not recommended for STEC infections.

Food is considered the major transmission vehicle of O157:H7 and, probably, many non-O157 STEC. Beef products (Bell, Goldoft et al. 1994) in particular, as well as dairy products (Upton and Coia 1994; Goh, Newman et al. 2002; Allerberger, Friedrich et al. 2003), fruits and vegetables (Fukushima, Hashizume et al. 1999) have all been implicated in STEC outbreaks or sporadic cases. Contaminated water has been reported as causing at least 49 O157:H7 outbreaks and 7 non-O157 outbreaks (Kasper et al. 2010). Direct contact to animals or infected people has recently become an important route for STEC transmission (Akiba, Kimura et al. 2005; Hanna, Humphreys et al. 2007). Person-person contact was especially mentioned as the most common transmission route among reported non-O157 STEC infections (Kasper et al. 2010).

STEC infection in cattle

Ruminant animals, especially cattle, are considered the principle reservoirs of STEC O157:H7, and appear to be reservoirs of most non-O157 STEC as well. The prevalence of O157:H7, in published reports of the past 30 years, ranged from 0.3 to 27.3% in beef cattle and from 0.2 to 48.8% in dairy cattle; the corresponding prevalence of non-O157 STEC ranged from 4.6 to 55.9% and from 0.4 to 74.0%, respectively (Hussein and Sakuma 2005; Hussein 2007).

Experimental infections with STEC, even with inoculum doses as high as 10^{10} CFU, are commonly nonpathogenic to healthy cattle, but occasionally result

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in transient nonhemorrhagic diarrhea in calves (Cray and Moon 1995). However, neonatal calves are considered susceptible to, at least, O157:H7 infections (Dean-Nystrom, Bosworth et al. 1997).

Stx1 produced by STEC has been previously showed to bind the lower crypt bovine epithelium in the jejunum, ileum and cecum (Hoey, Sharp et al. 2003; Schuller, Frankel et al. 2004). Stx2, possessing higher cytotoxicity, was not detected in these tissues, indicating bovine intestinal epithelium may not express the Stx2-specific Gb3 receptor isoform (Schuller, Frankel et al. 2004). Stx1 bound to bovine epithelium was eventually delivered to endosomal/lysosomal compartments, resulting in toxin inactivation (Falguieres, Mallard et al. 2001). Together with the fact that bovine endothelial cells do not express Gb3 receptors (Pruimboom-Brees, Morgan et al. 2000), the above may explain the resistance of cattle to STEC-induced diseases.

Organisms shed in bovine feces are considered the ultimate source of STEC contamination. Under natural infection, the concentration of O157:H7 in cattle feces was showed to range from 2×10^2 to 8.7×10^4 CFU/g, with individual animals continuously testing positive for the organism in feces for 1 to 16 weeks (Shere, Bartlett et al. 1998). In another survey conducted at a breeding farm in Japan (Widiasih, Ido et al. 2004), fecal shedding of O157:H7 lasted from less than 1 week to 10 weeks, with the concentrations varying from 4 to higher than 1.1×10^5 CFU/10g feces, and shedding of O26 lasted from less than 1 week to less than 3 weeks, with the concentration ranging from 3 to 2.4×10^3 CFU/10g. A

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similar magnitude of fecal shedding was also reported in another study (Robinson, Wright et al. 2004).

A subset of naturally infected cattle excreted larger quantities of O157:H7, higher than 10^4 CFU/g feces (Chase-Topping, Gally et al. 2008), compared with most other individuals in the population. These individuals are defined as super-shedders or persistent-shedders, as the O157:H7 shedding magnitude was positively correlated with the duration of culture-positive status (Davis, Rice et al. 2006). Recent studies have included specific shedding duration in the definition, such as more than 3 consecutive months (Lim, Li et al. 2007; Carlson, Nightingale et al. 2009) or more than half of fecal samples consecutively testing positive for more than 4 months (Menrath, Wieler et al. 2010). Super-shedders typically comprise approximately 4% of the population according to a statistical model (Matthews, McKendrick et al. 2006), but may contribute to approximately 80% of O157:H7 transmission to uninfected cattle (Matthews, Low et al. 2006). Non-O157 STEC super-shedders were recently also reported (Menrath, Wieler et al. 2010).

To establish experimental infection, oral inoculation with high doses of approximately 10^8 to 10^{10} CFU of STEC is commonly preferred (Cray and Moon 1995; Besser, Richards et al. 2001) and is reported to result in fecal shedding patterns that are considerably variable among individuals and experiments. The shedding durations have been reported to range from 20 to 43 days (Sanderson, Besser et al. 1999), or in another study (Cray and Moon 1995), 7 to 20 weeks for calves less than 14 weeks old and 2 to 14 weeks for 1-year-old adult cattle. In

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one study (Cray and Moon 1995), most calves that received 10^{10} CFU of O157:H7 had fecal shedding concentrations of 10^4 to 10^7 CFU/g during the first four days post-inoculation (PI), with several calves shedding at an extremely high level (approximately 10^8 to 10^9 CFU/g) in first two days PI. The shedding levels dropped sharply within the first two weeks PI to an undetectable shedding level within 30 days, with individual calves discontinuously shedding the organism at approximately 10^2-10^4 CFU/g thereafter throughout the 144-day experiment.

Persistent shedding is closely related to successful colonization of the intestinal mucosa, whereas organisms in intestinal contents could be quickly eliminated from animals. The recto-anal junction (RAJ) has been identified as a primary colonization site of O157:H7 in cattle (Naylor, Low et al. 2003), which is consistent with the observations that infection of O157:H7 in most bovine *in vivo* studies didn't cause apparent A/E lesions on the intestinal epithelium (Brown, Harmon et al. 1997) with the exception at the RAJ (Lim, Li et al. 2007); and colonization at the terminal rectum, especially the RAJ, is highly recognized among persistent shedding cattle. However, it is not clear whether the RAJ is also the primary colonization site for non-O157 STEC.

Many STEC adherence factors involved in the pathogenesis of human infection, such as the LEE, Saa, and some accessory adherence factors (Efa1, Iha, etc.), also contribute to the colonization in the bovine intestine (Dean-Nystrom, Bosworth et al. 1998; Stevens, van Diemen et al. 2002; Jenkins, Perry et al. 2003; Sheng, Lim et al. 2006). Studies identified several predominant PFGE-types of O157:H7 in persistent-shedders, possessing enhanced

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adherence ability to human intestinal cells *in vitro* (Cobbold, Hancock et al. 2007; Carlson, Nightingale et al. 2009). Moreover, phage type 21/28 of O157:H7 was associated with persistent shedding (Halliday, Chase-Topping et al. 2006). This phage type demonstrated altered regulation of the type III secretion system, which also resulted in up-regulated colonization of the organism (Chase-Topping, Gally et al. 2008).

Diet composition and feed regimen could also have an impact on STEC shedding. Low nutrient (Kudva, Hunt et al. 1997), easily fermented feed compositions (such as barley) (Bach, Selinger et al. 2005) and the practice of fasting animals during transportation (Kudva, Hatfield et al. 1995) were frequently linked to higher or more persistent STEC shedding in cattle. This is probably due to the effects of reducing the secondary fermentation in the large intestine, which could result in decreasing colonic volatile fatty acid (VFA) concentrations and increasing pH, providing a more hospitable intestinal environment for STEC survival and proliferation.

Efforts have been made to reduce the shedding level of STEC in cattle. Several STEC vaccines have been developed based on the immunization with bacterial proteins conferring the ability to colonize, including intimin (van Diemen, Dziva et al. 2007), Efa1 (Stevens, van Diemen et al. 2002; van Diemen, Dziva et al. 2007), EspA (Dziva, Vlisidou et al. 2007), H7 flagellin (McNeilly, Naylor et al. 2008) and LEE-coding secreted proteins (Potter, Klashinsky et al. 2004; Van Donkersgoed, Hancock et al. 2005). Administration of these vaccines successfully induced the host immune response with up-regulated IgA and/or IgG

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to various contents; however, only the vaccines with secreted protein preparation or purified H7 flagellin have thus far resulted in delayed shedding peak and/or reduced colonization rate, and their effect in reducing STEC prevalence in cattle population was limited (Van Donkersgoed, Hancock et al. 2005).

Providing probiotics also is a promising practice for reducing STEC shedding in ruminants. The use of various probiotics, including direct-fed microbials (Ohya, Marubashi et al. 2000; Lema, Williams et al. 2001; Brashears, Jaroni et al. 2003), competitive exclusion (Zhao, Doyle et al. 1998; Zhao, Tkalcic et al. 2003) and yeast culture (Liou, Sheng et al. 2009), have been reported to shorten STEC shedding period or significantly reduce the shedding concentration.

A pre-harvest diet shift from grain to forage was previously proposed to reduce pathogenic *E. coli* in cattle based on a research result that generic *E. coli* populations in grain-fed cattle were 1000-fold higher than in forage-fed cattle (Diez-Gonzalez, Callaway et al. 1998). The influence of forage or grain diet in STEC shedding has been under long-time debate with various studies showing conflicting results. On one hand, some results showed a sudden diet shift from grain to hay reduced O157:H7 prevalence in cattle populations (Keen et al., 1999). On the other hand, other studies indicated that experimentally inoculated forage-fed cattle shed O157:H7 for a longer duration with higher concentrations than grain-fed cattle (Kudva, Hatfield et al. 1995; Van Baale, Sargeant et al. 2004). Because forage quality, components, bacteria inoculation dose, or sampling methods were not standardized in these studies, caution should be taken before extrapolating or drawing conclusions from these results.

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Prevalence of antibiotic resistant bacteria in farm animals

Antibiotic resistance markers have been widely used for identifiable recovery of bacterial strains of interest from various environmental and animal samples, such as feces. Pre-inoculation selection of animals for experiments and the use of appropriate selective media or specific growth conditions commonly ensure the efficient use of these markers. However, in a recent study, the use of antibiotic resistance markers was confounded by similar resistance patterns exhibited by indigenous bacteria in cattle feces and the selection of calves free of bacteria that are resistant to specific antibiotics was not well satisfied, which indicates the prevalence of antibiotic resistant bacteria may be underestimated, especially under the extensive use of antibiotics in animal agriculture.

As recently as 2009, FDA released the first government report, which indicated approximately 29 million pounds of antimicrobial drugs had been sold or distributed for the use in food animals in the US in 2009 (FDA, 2010). The administration of antibiotics to livestock is defined by two major categories: for disease prevention and treatment under therapeutic levels; and, more extensively, for growth enhancement under non-therapeutic levels. Other than the benefits brought to the livestock production industry, antibiotics that are commonly added to feed or water at low concentrations could have a direct impact on intestinal microbial populations. Through long term selection pressure, a higher prevalence of antibiotic resistance genes in the intestinal microflora may result. To date, the outcomes associated with the extensive application of antibiotics have emerged as an important public health issue. In particular, efforts

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have been made to survey the prevalence of antibiotic resistant bacteria in animal fecal samples, as an indication of their prevalence in the intestinal microflora. It was reported in one study that 80.3% of 270 non-specific *E. coli* isolates and 97% of 101 *Salmonella* isolates collected from cattle feces, hides and carcasses were resistant to at least one antibiotic (Fluckey, Loneragan et al. 2007). High proportions of antibiotic resistant strains were also observed in *Campylobacter* isolates (60% of 448 isolates) derived from the feces of US feedlot cattle (Englen, Fedorka-Cray et al. 2005), in *Campylobacter* isolates (68.1% of 72 isolates) from poultry, beef and dairy cattle (Oporto, Juste et al. 2009), and in intestinal *E. coli* isolates (72.1%) from healthy pigs in a German production unit (Schierack, Kadlec et al. 2009). Among the resistant isolates in above studies, multidrug resistance was commonly observed. Isolates from livestock samples are frequently resistant to tetracycline, quinolones, ampicillin, chloramphenicol, kanamycin, streptomycin (Oporto, Juste et al. 2009; Schierack, Kadlec et al. 2009).

Bioluminescence

Bioluminescence is an enzymatically catalyzed luminescent phenomenon possessed by a remarkably diverse group of species in nature. Among this group, luminescent bacteria are the most abundant species. Light emission in bacteria is achieved by a luciferase-catalyzed oxidation of reduced flavin mononucleotide ($FMNH_2$) and long chain fatty aldehyde (RCHO) to flavin mononucleotide (FMN) and corresponding fatty acid, with the excess energy released in the form of blue/green light ($\lambda_{MAX} \approx 490$nm) (Meighen 1991). Both $FMNH_2$ and molecular

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oxygen are common metabolic products naturally available in living cells (Close, Patterson et al. 2010), and all of the other genes necessary for the luminescence reaction, including genes *luxAB* coding luciferase and genes *luxCDE* coding the fatty acid reductase complex for RCHO synthesis, are included within a single *luxCDABE* operon.

The majority of luminescent bacteria naturally exist in marine environments except *Photorhabdus*, which are symbiotic pathogens of terrestrial insects (Farmer, Jorgensen et al. 1989). Other than being once isolated from human wounds (Colepicolo, Cho et al. 1989), luminescent bacteria are seldom reported in animals. To date, bacterial bioluminescence has been widely used as a reporter in gene expression or as a whole cell biosensor. Unlike some other non-destructive live-cell image tools, interference from endogenous signals is much less of a problem with the use of bioluminescence. And compared to green (GFP) and red (DsRed) fluorescent proteins, bioluminescence has the advantanges of significant shorter incubation times and lower detection limits (Hakkila, Maksimow et al. 2002). Lux genes developed from *Vibrio fischeri*, *Vibrio harveyi* and *Photorhabdus luminescens* are currently the most well adapted bacterial lux systems. Luciferases from *Vibrio fischeri* and *Vibrio harveyi* prefer working temperatures of less than 30 ^{o}C and 37 ^{o}C, respectively. In contrast, luciferase from *Photorhabdus luminescens* can remain stable at temperatures as high as 45 ^{o}C ($t_{1/2}$ > 3h) (Szittner and Meighen 1990), which enables more flexible applications and is more suitable for studies conducted in animal models or expression of lux genes in eukaryotic cells.

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By fusing a complete lux operon (*luxCDABE*) or luciferase gene *luxAB* to the promoter of interest, the strength and regulation of gene expression at the transcription level can be measured in terms of luminescence intensity. In a similar way, the concentration and distribution of biluminescent transformed cells can be monitored. Utilization of *luxCDABE* biosensors has been reported in the monitoring of *E. coli* O157:H7 distribution and colonization on beef carcass surface tissues (Siragusa, Nawotka et al. 1999), infection of *Staphylococcus aureus* in antibiotic-treated or untreated mice (Francis, Joh et al. 2000), assessment of the invasion kinetics of *Salmonella enterica* Typhimurium in different eukaryotic cell lines (Flentie, Qi et al. 2008), and other studies. In practice, bioluminescent-marked bacteria have been used to evaluate the efficiency of newly developed antibiotics (Ulitzur 1986). Because a very low level of bioluminescence can be detected, bacteriophage with inserted *luxCDABE* are used to provide an early detection of foodborn pathogens in food or water samples (Griffiths 1993; Brigati, Ripp et al. 2007).

Green fluorescent protein

Biological fluorescence is generated from chromophore at an excited state. In the case of native Green Fluorescence Protein (GFP) from the jellyfish *Aequorea victoria*, upon being excited at 395nm wavelength, emits a bright green fluorescent light with a major peak at 508nm (Tsien 1998). The tri-peptide motif Ser65-Tyr66-Gly67 close to the center of protein structure spontaneously forms a chromophore with fluorescence-emission ability in the presence of molecular oxygen (Tsien 1998). This maturation process is independent of any *A. victoria*

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specific enzymes or cofactors, therefore fluorescence can be generated where GFP is expressed.

Similar to bioluminescence, GFP has been widely used in cell biology research to monitor gene expression, protein-protein interaction, protein localization in real-time and as a whole cell biosensor to detect, for example, pathogen distribution and concentrations. Compared to the other fluorescent dyes, GFP has a much lower cytotoxicity and was showed to have little impact on cell physiology or protein activity even when expressed at high levels (Chalfie, Tu et al. 1994). However, unlike bioluminescence, the use of GFP, especially as a whole cell biosensor, can be confounded by autofluorescence emitted from certain cellular molecules in the host or from some media when they are excited at appropriate wavelengths. Meanwhile, fluorescence is not enzymatically catalyzed; in other words, fluorescent light intensity is positively correlated with the number of mature chromophore and the level of GFP expression. To overcome background noise from endogenous fluorescence, GFP, when used as a biosensor of bacteria, is typically expressed from plasmids rather than from chromosomes, to accomplish higher expression levels. Considering the rare occurrence of indigenous fluorescence in human pathogens, GFP cell biosensor has been used in studies to determine the concentration and duration of survival, for example, *E. coli* O157:H7 and *Salmonella* Typhimurium in animal manure, soil (Himathongkham, Bahari et al. 1999; Franz, van Diepeningen et al. 2005), and plant tissues under different treatments. Stability of plasmid-borne GFP marker in bacteria might be questioned when there's no additional selection

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pressure being application, however, in the above studies, green fluorescence was persistently detected from plasmid-expressed GFP strains after inoculation in soil and manure for at least 80 days (Franz, van Diepeningen et al. 2005; Lagendijk, Validov et al. 2010). To our knowledge, however, most studies inoculated plasmid-expressed GFP pathogens directly to animal feces or other environmental samples. Few studies have tested the feasibility of using such GFP markers for the recovery of pathogens from feces after oral inoculation of animals and passed through the GIT, where they are subjected to stressful conditions from very diverse intestinal microflora and complicated physical conditions. This question will be tested in the current study.

Goals of this research

STEC has recently emerged as an important group of foodborne pathogens in many countries. As principle reservoirs of the organism, cattle are considered to be the ultimate sources of contamination. A previous study (Hussein 2007) estimated the prevalence of STEC among feedlot cattle ranged from 0.3 to 19.7% for O157:H7 and from 4.6 to 55.9% for non-O157 STEC serotypes. Although sanitary procedures during processing are effective in reducing STEC on carcasses (Elder, Keen et al. 2000), the organisms were still detectable from post-processing carcasses, retail products in supermarkets and fast food restaurants, with the prevalence rates of 0.01-43.4%, 0.1-54.2% and 2.4% respectively for O157:H7, and 1.7-58.0%, 3.0-62.5% and 3.0% respectively for non-O157:H7 (Hussein 2007).

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Serogroup O91, although seldom reported in outbreaks or HUS cases in the US, is among the five serogroups most frequently associated with human illnesses and outbreaks in Europe and is the most prevalent serogroup among adult patients in Germany (Mellmann, Fruth et al. 2009). A survey (Werber, Beutin et al. 2008), that reviewed samples collected from 1996 to 2007 in Germany, ranked O91 as the second most prevalent serogroup (6% of 448 isolates from foods) in food samples and the fourth among isolates from patients (10% of 1447 isolates). Interestingly, in the same study, O91 was the only serogroup frequently isolated from both food samples and patients (Werber, Beutin et al. 2008). Within the serogroup, O91:H21 is the only serotype implicated in HUS cases (Oku, Yutsudo et al. 1989; Werber, Beutin et al. 2008). Genetic analysis of an O91:H21 isolate revealed the presence of a Stx2 variant, Stx2vh, which is considered to be an indicator of potential severe consequences in human infection (Zweifel, Giezendanner et al. 2010). Oral infection in a streptomycin-treated mouse model also revealed the high virulence of this serotype, with two O91:H21 isolates having LD_{50} less than 10 bacteria (Lindgren, Melton et al. 1993).

An enhanced understanding of heterogeneous shedding patterns of STEC by cattle and factors that might influence shedding magnitude and duration could help develop strategies to reduce the level of STEC entering food production systems, and, therefore, control infections resulting from the consumption of STEC contaminated foods. Seasonal shedding patterns have been well documented previously. Reports indicate that the prevalence of STEC in cattle

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populations commonly reach the highest rates in the summer and decreases to very low rates during the winter (Hancock, Besser et al. 1997; Pradel, Livrelli et al. 2000; Barkocy-Gallagher, Arthur et al. 2003; Miller, Beasley et al. 2003). This is consistent with the seasonal patterns of STEC infection in human, where greater incidence of illnesses occurs during the late summer months (Rangel, Sparling et al. 2005; Friesema, J et al. 2010). However, the mechanisms of seasonality are not clear. Among various factors distinct between seasons, one study (Edrington, Callaway et al. 2006) showed positive correlation between day length and O157:H7 prevalence in cattle feces ($r = 0.67$; $P = 0.0009$) as well as between ambient temperature and O157:H7 prevalence ($r= 0.43$; $P = 0.05$).

Previously in our experiment, a study was conducted to determine the effect of controlled warm and cool ambient temperature on shedding properties of STEC O157:H7 and O91:H21 in calves. Holstein steers were assigned to an oral inoculation of O157:H7 (n=4) or O91:H21 (n=4). Within calves inoculated with the same strain, half were housed under a cool temperature regimen (12h at 18°C; 12h at 15°C) and the other half under a warm temperature regimen (12h at 32°C; 12h at 27°C). The concentration of each inoculated strain contained in feces was examined every two days for up to 2 weeks PI. Sorbitol McConkey agar (SMAC) containing 50ug/ml nalidixic acid was used to recover strains from fecal samples, since both strains are resistant to this antibiotic. However, after 10 days PI, isolates recovered in fecal samples from some calves were genetically inconsistent with initial challenge strains, and these interfering bacteria were not distinguishable from the challenge strains via colony morphometrics. Similar

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contaminating bacteria were also isolated from candidate calves originally intended for a second trial but that had not been subjected to inoculation. Four dominant types of colonies with distinct phenotypes (color and size of the colony) were observed among the contaminating bacteria from feces of candidate calves on the selective SMAC agar. Two colonies of each phenotype were tested for their identity based on the results of 20 biochemical reactions included on the API 20E strip (BioMerieux Vitch, Inc., Hazelwood, MO). According to the interpretation of API 20E database, majority of the tested colonies (6 colonies belonging to 3 phenotypes) were characterized as *E. coli* with very high identification and the remaining two colonies were characterized as *E. coli* with great likelihood but low selective identity. Using the NARMS CMV1AGNF panel (Trek Diagnostics, Cleveland, OH), all four phenotypes of colonies demonstrated multi-drug resistance (Table 1), which confirmed the difficulty for the use of antibiotic resistance markers.

Thus, to address what appear to be significant shortcomings of antibiotic resistance markers for STEC challenge studies where the presence of multi-drug resistant indigenous bacteria might confound such, the objective of this study was to develop more effective markers suitable for research on STEC using animal models or environmental samples. Chromosomally-integrated *luxCDABE* and plasmid-borne GFP markers of STEC were developed and evaluated both in *vitro* in terms of growth cost, stability, and other relevant aspects, and in O91:H21 shedding experiment to determine their feasibility in cattle challenge experiments.

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Table 1. Four antibiotic resistance profiles[a] displayed by four dominant phenotypes of interfering fecal isolates from candidate calves prior to inoculation

	1	2	3	4
Cefoxitin	4 (S)	4 (S)	4 (S)	16 (I)
Amikacin	16 (S)	2 (S)	2 (S)	2 (S)
Chloramphenicol	>32 (R)	32 (R)	32 (R)	>32 (R)
Tetracycline	>32 (R)	>32 (R)	>32 (R)	>32 (R)
Ceftriaxone	≤0.25 (S)	≤0.25 (S)	≤0.25 (S)	≤0.25 (S)
Amoxicillin/Clavulanic Acid	8/4 (S)	8/4 (S)	8/4 (S)	2/1 (S)
Ciprofloxacin	>4 (R)	>4 (R)	>4 (R)	0.5 (S)
Gentamicin	4 (S)	4 (S)	1 (S)	8 (I)
Nalidixic acid	>16 (R)	>16 (R)	>16 (R)	>16 (R)
Ceftiofur	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)
Sulfisoxazole[b]	>256	>256	>256	>256
Trimethoprim/sulfamethoxazole	>4/76 (R)	>4/76 (R)	>4/76 (R)	>4/76 (R)
Kanamycin	>64 (R)	>64 (R)	>64 (R)	>64 (R)
Ampicillin	>32 (R)	>32 (R)	>32 (R)	>32 (R)
Streptomycin	64 (R)	64 (R)	>64 (R)	>64 (R)

[a]S – Susceptible, I – Intermediate, R – Resistant. Susceptibility of isolates was determined by Minimum Inhibitory Concentrations (ug/ml) of each antibiotic. Antibiotics contained on NARMS plates were all listed here.
[b]256ug/ml is the highest concentration for sulfisoxazole on NARMS plate. Based on CLSI standard that MIC breakpoint for resistance to sulfonamide antibiotics is 512ug/ml, sulfisoxazole resistance cannot be determined.

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MATERIALS AND METHODS

Bacterial strains

STEC O91:H21

The STEC O91:H21 strain used in this study was provided by our collaborator, Dr. Nora Lia Padola of the Faculty of Veterinary Sciences, University Nacional del Centro PBA, Tandil, Argentina. It was originally isolated from a cattle farm in Argentina and was confirmed to harbor *stx2* (shiga toxin type II), *saa* (autoagglutinating adhesin) and *ehxA* (enterohemolysin) genes. Besides the resistance to nalidixic acid, the strain was also confirmed to be resistant to trimethoprim and erythromycin with minimum inhibitory concentrations (MIC) of 125ug/ml and 250ug/ml, respectively. The two antibiotics were previously used in combination with nalidixic acid in SMAC selective medium for additional selection efficiency with the highest concentrations at 50ug/ml, 100ug/ml, respectively; however the purpose of eliminating indigenous bacteria was not well satisfied.

Construction of bioluminescent E. coli O91:H21

Construction of a bioluminescent *E. coli* O91:H21 was achieved by conjugation followed by a suicide plasmid delivery of the *luxCDABE* operon into the recipient strain and transposon mutagenesis. The suicide plasmid carried by the donor strain *E. coli* S17/pUT mini-Tn5 *luxCDABE Km2* (a gift from Dr. David Piwnica-Worms, Washington University School of Medicine, St. Louis, MO)

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contained the *luxCDABE* operon engineered from *Photorhabdus luminescens* and a kanamycin resistance gene downstream of *luxE* gene, which together are flanked by mini-Tn5 transposon components (Winson, Swift et al. 1998). The O91:H21 isolate from Argentina was used as the recipient strain. Conjugation followed the procedure described previously (Winson, Swift et al. 1998), with some modifications. Briefly, cells harvested from overnight cultures were gently washed twice to remove antibiotic residues and then resuspended in 1/10 of original amount of LB broth (Fisher Scientific, Fairlawn, NJ). Donor and recipient cells were well mixed in equal ratio and spot inoculated on LB agar plates. Upon inoculation at 37°C for 7 h, cells were scraped from LB plates and spread on LB agar plates containing 50 ug/ml nalidixic acid and 50 ug/ml kanamycin for the selection of transconjugants. Three isolates showing the brightest light visible by naked eye in the dark were saved as candidates and were designated as *E. coli* O91:H21/Lux1, Lux2 and Lux3. Along with these isolates, 7 other isolates with less intense bioluminescence were also preserved. The absence of the transfer plasmid was confirmed for all isolates by a susceptibility test to 100 ug/ml ampicillin (the ampicillin resistance gene is located on the transfer plasmid, but outside of mobile element) and plasmid extraction followed by electrophoresis.

Construction of GFP-transformed E. coli O91:H21

Construction of the GFP-transformed *E. coli* O91:H21 was achieved through the transformation of a pGFP vector (Clontech Laboratories, Inc., CA) containing ampicillin resistance gene into competent O91:H21 cells prepared via

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the Calcium Chloride method (Mandel and Higa 1970; Cohen, Chang et al. 1972). Successful transformation conferred growth ability on LB agar containing 100 ug/ml ampicillin and 50 ug/ml nalidixic acid, along with emission of visible strong green light when colonies were exposed to UV light in the dark.

In vitro validation of Lux- and GFP- constructs

API 20 E test

API 20 E strip (BioMerieux Vitch, Inc., Hazelwood, MO) was used according to the manufacturer's instructions to determine whether the integration of *luxCDABE* affected the biochemical reactions of the original organism included on the strip. The color of each well representing each biochemical reaction was compared between the three lux-constructs and the original O91:H21 strain.

Confirmation of essential genes by multiplex PCR

Random transposon integration may result in the disruption of a gene conferring STEC virulence. To ensure against such, all three Lux-integrated constructs were tested by two multiplex PCRs (M-PCR) for the presence of genes *stx2*, *saa* and *ehxA*, as well as genes *wbsD* and *fliCH21* that represent antigens unique to serotype O91:H21. Primers used in all PCR reactions of this study are listed in Table 2. Total DNA was prepared by boiling cell pellets, harvested from overnight culture in sterile ddH_2O, for 10 min followed by centrifugation at 13,000 rpm. Supernatant containing whole genomic DNA was immediately used for PCR. The M-PCR conditions for the detection of *stx2*, *saa*

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and *ehxA* genes were: 1) 1 cycle of 94°C for 5 min; 2) 35 cycles of 94°C for 30 s, 57°C for 1 min and 72°C for 1 min 30 s; 3) 1 final cycle of extension at 72°C for 5 min. The conditions for *wbsD* and *fliCH21* amplification were: 1) 1 cycle of 94°C for 5 min; 2) 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; 3) 1 final cycle of 72°C for 7 min. Each reaction included 12.5 ul GoTaq® Green Master Mix, 2X (Promega, Madison, WI), 1 ul of each primer (5 pmol/ul), 1 ul template DNA and 5.5 ul sterile ddH_2O. All reactions were conducted in a Mastercycler Gradient thermocycler (Eppendorf, Westbury, NJ).

Identification of transposon insertion site

In order to better characterize the three Lux-integrated constructs, a Touchdown PCR previously described (Levano-Garcia, Verjovski-Almeida et al. 2005) was used to identify transposon insertion sites in the chromosome. A hybrid-consensus degenerated primer, called HIB17, targeting unknown regions and an outward facing primer Kan specifically targeting the 3' end of kanamycin resistance gene located immediately downstream of *luxE* were used. PCR reaction conditions were as previously described, except GoTaq® Green Master Mix (Promega, Madison, WI) was used. After separating PCR products on the gel, products exhibiting the brightest bands were purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced by the Molecular Biology Resource Facility at The University of Tennessee (Knoxville, TN).

Identical sequence results were found between O91:H21/Lux2 and Lux3, indicating they were from the same clone. Sequencing the PCR product of

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O91:H21/Lux1 was not successful. Therefore, only O91:H21/Lux2 was used in the following experiments. Alignment of the sequence of O91:H21/Lux2 with the complete genome sequence of *Escherichia coli* CFT073 revealed that the transposon insertion site was approximately next to the *yihM* gene. A new primer, Yih, was designed for targeting the 5' end of *yihM* gene. The PCR product amplified by primer yih and luxE (Flentie, Qi et al. 2008) targeting 3' end of *luxE* gene was sequenced and the exact transposon insertion site was determined by localization of the conserved I-end sequence (5'-CTGTCTCTTGATCAGATCT-3') of mini-Tn5 transposon.

Growth curves

Growth curves were measured for *E. coli* O91:H21, O91:H21/Lux2 and O91:H21/GFP constructs. Strains were grown to mid-log phase (OD_{600}≈0.8) with very similar OD_{600} values (±0.01). Each culture was diluted 1:100 in fresh LB broth, and incubated at 37°C with shaking at 200 rpm. Growth was measured in terms of OD_{600} value of the culture every hour for the first 10 hours and every 2 hours for the next 10 hours. The experiment was conducted in duplicate.

Growth data of three strains were analyzed using Graphpad Prism software (GraphPad Software, Inc., La Jolla, CA). Growth curves were generated using OD_{600} values according to the modified nonlinear *Gompertz* model (Fig. 1) (Zwietering, Jongenburger et al. 1990; Romano, Molla et al. 2009) and the best-fit values of the parameters in *Gompertz* equation, including maximal growth rate (μ_{max}), lag time (λ) and initial bacterial number (N_0) and the difference between

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final and initial bacterial number (A) were compared between three groups using F-test.

In vitro stability assay

The stability of integrated *luxCDABE* and transformed GFP gene was tested by subculturing two O91:H21 constructs in liquid medium without antibiotic selection. For each passage, a 20ul aliquot from the previous passage was inoculated into 5 ml of fresh LB broth without antibiotics. The culture was passed after every 12 h for up to 60 passages (approximately 2160 generations if the generation time of our *E. coli* strains was 20 min), unless a significant loss of the marker gene was observed.

Samples from each subculture were tested on LB agar containing appropriate antibiotics to detect the presence of luminescence-negative or green fluorescence-negative isotypes during the first 10 passages and from every five subcultures thereafter. To identify luminescence-negative colonies on LB agar plates, a FluorChem® imaging system (Alpha Innotech Corp, San Leandro, CA) was used. Two images were taken for each plate with one taken under artificial white light (Fig. 2A) and the other in the dark (Fig. 2B). Luminescence-negative colonies which were only displayed in the image taken with light were further subject to M-PCR for their isotypes to determine whether they were the isogenic O91:H21 without bioluminescence marker. The presence and percentage of green fluorescence-negative colonies in subculture population were determined

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by exposing colonies to UV light, and fluorescence-negative colonies were tested for the isotypes to ensure the confidence of stability results.

Animal experiments

Calves, housing and treatment

The shedding experiment of wild type O91:H21 was conducted separately from those of *luxCDABE* and GFP-marked strains and was a part of the previous shedding experiment where nalidixic acid resistance marker was used for identifiable recovery.

For the shedding experiment of *luxCDABE* and GFP-marked strains, eight Holstein steers with average body weight of 85.6±8.1 kg were obtained from a private commercial dairy farm in Kentucky and were transported to and maintained in The Joe Johnson Animal Research and Teaching Unit (JARTU) of the UT AgResearch East Tennessee Research and Education Center near the Knoxville campus. Calves were assigned to one of four identical BSL2 containment rooms, with two calves maintained in each room separated by metal pens to avoid physical contact. Each pen was equipped with an independent water system and a feeding trough by which animals were provided *ad libitum* access to feed and water. Animal rooms were cleaned with water via large delivery volume hose every day.

Following 5 days of acclimation to the rooms, four calves from two rooms received the O91:H21/Lux2 challenge strain and the four calves in the other two

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rooms received the O91:H21/GFP. To prevent cross-contamination, coveralls and gloves were changed and boots disinfected in a boot wash between rooms of the two challenge strains.

Bacteria preparation and inoculation

Prior to inoculation into the calves, both the *luxCDABE*-integrated and GFP-transformed O91:H21 constructs were tested for their ability to survive in bovine feces via stab inoculation of the organisms in unsterile feces. After overnight incubation at 37°C, the two strains were successfully recovered with different selective media, including LB containing 50 ug/ml kanamycin and 50 ug/ml nalidixic acid for lux-construct and LB containing 100 ug/ml ampicillin and 50 ug/ml nalidixic acid for GFP-construct.

One loopful of O91:H21/Lux2 and O91:H21/GFP cultures from the original stock was inoculated into 100 ml LB broth and incubated overnight at 37°C. Bacterial concentrations were determined via serial dilution and plating on LB agar. Enumeration revealed a concentration of 6.5 x 10^{8} CFU/ml for O91:H21/Lux2 inoculum and 2.3 x 10^{8} CFU/ml for O91:H21/GFP.

Twenty milliliters of bacterial culture was orally administered to each calf through an esophageal feeding tube. With the tube still in position in the animal, an additional 200ml of sterile LB broth was applied to wash residual bacteria in the tube into the calf.

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Sample collection

Fecal samples were collected on the day calves arrived, immediately before inoculation and every two days PI for a period of two weeks. Fresh fecal samples were obtained by rectal palpation. In cases where animals were seen to defecate during pen cleaning during a sampling period, fresh fecal material was immediately collected from the evacuated stool on the floor, avoiding touching the tube to contaminating surfaces or older manure. Five to ten grams of each sample were collected using a sterile 50ml plastic tube, and transported to the laboratory immediately in ice. At the end of the trial, all animals were euthanized via IV injection of pentobarbital sodium (Beuthanasia D, Schering-Plough Animal Health, Union, N.J) delivered at 10 ml per 100 lb of animal body weight. Samples were collected from five locations of intestine, including the duodenum, ileum, cecum, and descending colon and rectum. All animal and biosafety protocols were approved by the University of Tennessee Institutional Animal Care and Use Committee, and BioSafety Committee, respectively.

Bacterial Enumerations

Approximately two grams of each manure sample was diluted to 20% (wt./vol.) in 1x phosphate buffered saline (PBS) and well mixed via vigorous vortexing. Mixtures were maintained at room temperature for 30min for precipitation of solid matter. The supernatant was then serially diluted, and 100ul of each dilution was spread on LB plates containing 50 ug/ml nalidixic acid and one other antibiotic (50 ug/ml kanamycin for O91:H21/Lux2 treatment and 100

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ug/ml ampicillin for O91:H21/GFP treatment). The detection limit by the plating procedure described above is 50 CFU/g feces.

In practice, counting the luminescent colonies by naked eye in the dark posed some difficulty, especially when the number of colonies was large. To overcome this inconvenience, the previous mentioned method using FluorChem® imaging system (Alpha Innotech Corp, San Leandro, CA) was used for enumeration. Bioluminescent colonies were enumerated from the image taken in the dark. Additionally, both the bioluminescence-positive and negative colonies were selected for M-PCR testing of isotypes to determine the confidence of enumeration results. For a convenient separation of the two types of colonies, especially on a cluttered plate, the two images of each plate taken by FluorChem® imaging system were superimposed using the Overlay function in AlphaEase® FC software (Alpha Innotech Corp, San Leandro, CA) to provide the exact position of each bioluminescent colony (Fig. 1C). Green fluorescent colonies were enumerated by exposing colonies to UV light.

Colony confirmation

DNA prepared directly from individual colonies was subjected to M-PCR for the presence of *stx2*, *saa* and *ehxA* genes. Within the group receiving lux-O91:H21, up to 20 bioluminescent colonies from each sample were selected for M-PCR to confirm the isotype. Bioluminescent-negative colonies from SMAC plates containing 50 ug/ml nalidixic acid and LB plates containing both nalidixic acid and kanamycin were also tested for the isotype to determine whether the

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loss of *luxCDABE* operon occurred during the experiment. Additionally, bioluminescent-positive colonies from LB plates without antibiotics were also tested to determine whether there's a chance for the mini-Tn5 transposon to jump to and generate luminescence in co-habitating bacteria.

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Table 2. Primers used in this study

Primer	DNA Sequence (5' to 3')	Product size (bp)	Reference
Stx2-F	CTTCGGTATCCTATTCCCGG	516	(Blanco, Blanco et al. 2004)
Stx2-R	CTGCTGTGACAGTGACAAAACGC		
Saa-F	CGTGATGAACAGGCTATTGC	119	(Paton and Paton 2002)
Saa-R	ATGGACATGCCTGTGGCAAC		
EhxA-F	GGTGCAGCAGAAAAAGTTGTAG	1551	(Schmidt, Beutin et al. 1995)
EhxA-R	TCTCGCCTGATAGTGTTTGGTA		
WbsD-F	GCTGACCTTCATGATCTGTTGA	291	(Perelle, Dilasser et al. 2002)
WbsD-R	TAATTTAACCCGTAGAATCGCTGC		
FliCH21-F	ACAGGATAAAGATGGCAAACAAGTT	108	(Auvray, Lecureuil et al. 2008)
FliCH21-R	GCAGCCACTGCAAGCTTAGTT		
HIB17	CGGAATTCCGGATNGAYKSNGGNTC	N/A	(Levano-Garcia, Verjovski-Almeida et al. 2005)
Kan	CGAGCTCGAATTCGGCCTAG	N/A	(Flentie, Qi et al. 2008)
LuxE	TGAGGATGAAATGCAGCGTA	N/A	(Flentie, Qi et al. 2008)
Yih	CGACGGCTTCGTCAATAGAT	N/A	This study

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RESULTS

Characterization of *luxCDABE*-integration and GFP-transformed STEC O91:H21

The integration of *luxCDABE* operon apparently did not disrupt the genes conferring the production of O91, H21, Stx2, Saa or enterohemolysin, as PCR products of these genes were of identical sizes between the otherwise isogenic STEC O91:H21 with and without *luxCDABE*. No significant color difference was observed from each well of the API 20e strip (Fig. 3), suggesting the transposon integration and the production of luciferase and its substrates did not impact the biochemical reactions included in API 20e system.

The transposon integration site on the chromosome was further determined using a previously mentioned touchdown PCR procedure (Levano-Garcia, Verjovski-Almeida et al. 2005). According to the *E. coli* strain CFT073 complete genomic sequence annotation, the *luxCDABE* operon was integrated at nucleotide 4,586,515 (Fig. 4A and 4B). The integration site was 27 nucleotides before the stop codon of gene *yihL*, which is a putative transcriptional regulator (Welch, Burland et al. 2002) and may be involved in the transcription of downstream gene *yihM* coding a putative sugar phosphate isomerase (Welch, Burland et al. 2002). Currently, little is known about the role of gene *yihL* or *yihM* in *E. coli*. Although another two genes, *yihO* and *yihQ*, within the *yih* operon of *Salmonella* were involved in O-antigen capsule assembly and translocation

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(Gibson, White et al. 2006), it is not known whether the role of *yihL* and *yihM* genes within the *yih* operon of *E. coli* is related to similar functions as well.

Growth curves of O91:H21 strain and two constructs with *luxCDABE* and GFP markers were compared via the comparison of parameters in the *Gompertz* equation. No significant difference was found in the maximum growth rate (P=0.5446), lag time (P=0.3267), initial OD_{600} value (P=0.3875) and maximum OD_{600} difference (P=0.3487) among three groups, suggesting the fitness of lux-marked and GFP-marked constructs was not affected by the introduction of chromosome-integrated *luxCDABE* and plasmid-borne GFP markers in pure culture.

The strain containing the transposon integrated *luxCDABE* marker was subcultured in liquid medium for 60 passages. During this *in vitro* stability assay, only one bioluminescence negative colony was observed at the eighth passage. All other colonies were tested positive for bioluminescence with similar intensity and resistance to kanamycin.

In contrast, the *in vitro* stability assay indicated the plasmid-bearing GFP marker had low stability in our O91:H21 strain. GFP-negative colonies were first detected at the fourth passage, comprising approximately 0.63% of the population. After that, the percentage of GFP-negative isotypes in the population of each subculture started to increase slowly to 3.45% at the eighth passage, and then increased dramatically to 54.21% at the tenth passage. Therefore, the

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approximate half-life of this GFP-marked O91:H21 in liquid medium was 10 passages.

Fecal shedding of STEC O91:H21 in experimentally infected calves

All calves receiving either the *luxCDABE*-marked, GFP-marked or wild type O91:H21 remained physically healthy with normal appetites throughout the two-week shedding experiment. Within all three treatment groups, 1 to 2 calves of each group were observed exhibiting non-bloody diarrhea intermittently. No bloody diarrhea was observed.

Wild type O91:H21

Four calves were initially used to assess the fecal shedding pattern of wild type O91:H21. Due to the presence of sorbitol-fermenting and nalidixic acid resistant indigenous bacteria in one calf at the beginning of the experiment, the data recorded for this individual were not used.

As showed in Fig. 5, the number of wild type O91:H21 shed in bovine feces was 1.4×10^3, 6.4×10^3 and 1.8×10^4 CFU/g, respectively, in three calves at day 2 PI (first sampling point after inoculation). One calf shed the organism intermittently, however it was not known whether the organism was absent from cattle intestine at day 10 PI where the fecal sample was tested negative of the organism. Only one calf was continuously tested positive of the organism for a

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two-week period, while the remaining calf continuously shed the organism for 12 days and was tested negative of the organism in feces at day 14 PI. As mentioned previously, in two of the three calves that received wild type O91:H21, post-experiment M-PCR analysis showed the presence of indigenous bacteria, which was recovered from fecal samples collected after day 10 PI, on selective plates. Because these interfering organisms were unable to be separated from challenge strain on SMAC plate containing 50ug/ml nalidixic acid, the enumeration results would include the number of these organisms and, therefore, could not accurately reflect the shedding magnitude.

Contents from four different parts of the bovine intestine, in addition to the feces from rectum, were collected at the end of the experiment and tested for the presence of O91:H21 (Fig. 7). The organism was not isolated from duodenal contents of any calves. Two calves, that were tested positive for the organism in feces at day 14 PI, were also tested positive for the organism in the contents of cecum and colon. The organism also was found in the ileum of one of the two calves, while ileal content from the other calf was not available during the sampling. Although the organism was not detected in feces from the remaining calf, the organism was recovered from ileum and colon. As mentioned above, the estimation of concentrations of the organism in intestinal contents of two calves was confounded by the presence of indigenous bacteria with nalidixic acid resistance and was not precise.

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LuxCDABE-marked O91:H21

As showed in Fig.8, the fecal count of *luxCDABE*-integrated O91:H21 was 1.2×10^3, 1.2×10^3, 8.7×10^3 and 1.3×10^4 CFU/g, respectively among four calves at day 2 PI. Among the four calves, two with lower initial shedding levels became undetectable at day 12 and 14 PI, respectively, while the shedding levels in the remaining two calves with higher initial shedding levels decreased to 5.8×10^2 and 8.7×10^2 CFU/g, respectively at day 14 PI.

The screening for bioluminescence-positive O91:H21 in contents of duodenum, ileum, cecum and descending colon of all calves at day 14 PI (Fig. 9) showed no such bacteria were detected in duodenal contents collected from all calves. In the two calves that maintained detectable fecal counts at day 14, the organism was recovered from the contents of the other three parts of the GIT, with the concentrations of 3.0×10^2 and 5.0×10^1 CFU/g in ileum, 6.3×10^2 and 1.3×10^3 CFU/g in cecum, 1.0×10^3 and 1.0×10^3 CFU/g at descending colon, respectively. In the other two calves that did not shed detectable levels of the organism in feces at day 14, the organism was still detectable by plating from the contents of cecum and descending colon with concentrations at approximately 5.0×10^1 CFU/g.

During the two-week shedding experiment, selected luminescence-positive and negative colonies recovered from each fecal samples were tested for isotype to assess the stability of the marker under conditions related to the

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presence of indigenous gut microflora. A total of 242 bioluminescence-positive and 81 bioluminescence-negative colonies from LB agar containing 50 ug/ml nalidixic acid (resistance feature of lux-marker host strain) and 50 ug/ml kanamycin (resistance feature carried with *luxCDABE* marker) as well as 71 bioluminescence-negative colonies resistant only to nalidixic acid were tested. All the positive colonies were confirmed as the lux-marked O91:H21 inoculum while all the negative colonies belonged to indigenous microflora, suggesting that the *luxCDABE* marker was stably maintained in host strain and could efficiently distinct marked strain from indigenous bacteria and, therefore, ensured the reliability of the enumeration results of the cattle shedding experiment.

GFP-marked O91:H21

Stability of the plasmid-bearing GFP marker was poor during the assessment of fecal shedding pattern of this construct in calves. No GFP-positive colony was recovered from fecal samples of any calves receiving GFP-marked O91:H21 throughout the sampling period. However, isogenic GFP-negative O91:H21 isolates were detected from all four calves using SMAC agar containing 50 ug/ml nalidixic acid and confirmed by M-PCR analysis, indicating the challenge strain had successfully localized in calf intestine or intestinal contents but the plasmid carrying the GFP marker was not well maintained in the construct in the presence of indigenous microflora and other conditions within the intestine. Although the estimation of shedding magnitude could be very inaccurate as

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confounded by the presence of indigenous bacteria exhibiting sorbitol fermenting and nalidixic acid resistant features, our results showed that two of the four calves shed the O91:H21 strain for the entire two-week sampling period, while the other two did not shed detectable levels of the organism at day 6 and day 10 PI, respectively.

No O91:H21 was recovered from the duodenal contents in calves that received GFP-marked O91:H21. Contents of the ileum, cecum and descending colon tested positive for O91:H21 in the two calves with fecal shedding for at least 14 days; however, only the contents of the cecum and descending colon were positive for the organism in the calf that shed the organism for at least 10 days. No O91:H21 was recovered from intestinal contents of the remaining calf.

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DISCUSSION

The shedding magnitude and duration of STEC in ruminant feces is an important topic, particularly because of the relevance to various STEC contamination sources and significant numbers of human infections. However, the traditional way of using antibiotic resistance markers for the identifiable recovery of the inoculated STEC strains from cattle feces can be interfered by indigenous microflora exhibiting similar antibiotic resistance patterns, as observed in our previous study. In this study, a chromosomally-integrated *luxCDABE* based bioluminescence marker system was developed in STEC O91:H21 and showed reliability for the detection and enumeration of STEC and was used to monitor the fecal shedding of O91:H21 for a two-week period. On the other hand, a plasmid-borne green fluorescent protein marker showed poor stability in the same O91:H21 host strain and is not recommended for long term experiments without appropriate antibiotic selection (assuming such is possible).

Several recent studies (Englen, Fedorka-Cray et al. 2005; Fluckey, Loneragan et al. 2007; Schierack, Romer et al. 2009) have reported a high proportion of isolates, including *E. coli*, *Salmonella*, and *Campylobacter*, from fecal microflora of farm animals were resistant to at least one antibiotic, with a portion of the isolates exhibiting resistance to multiple antibiotics. For many shedding experiments, the traditional way of using antibiotic resistances as markers for the recovery of marked challenge organisms from animal or

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environmental samples requires rigorous pre-inoculation screening to ensure the test animals are free of bacteria resistant to selective antibiotics; otherwise, the indigenous microflora exhibiting antibiotic resistance may confound the detection and enumeration of the marked challenge organisms using antibiotic-supplemented selective media. In some circumstances, as observed in the shedding experiment of wild type O91:H21 in our previous study, animals confirmed free of interfering bacteria prior to inoculation may obtain such bacteria later during the experiment from environment, feed and water, which could greatly confound enumeration results and affect the entire experiment.

Among STEC, specially designed selective or differential media are available for typical O157:H7 based on its unique phenotypes including delayed or defective sorbitol fermentation and negative β-glucuronidase (GUD) activity. The use of such media could help enhance the selection of this organism from animal feces, as reported in several studies (Kudva, Hatfield et al. 1995; Sheng, Davis et al. 2004). As for non-O157 STEC, few effective selective media are currently available due to the high genetic diversity of these organisms. Some commonly-used selective media for non-O157 STEC, such as MacConkey (MAC) or SMAC agar, even in combination with selective antibiotics, may not provide adequate discrimination between indigenous resistance bacteria and antibiotic resistance marked challenge organisms, as observed in this study.

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The bioluminescence marker and green fluorescence marker, due to their rare phenomenon in livestock microflora, have the potentials to complement the inadequate selection of antibiotics and/or selective media in shedding experiment.

To fulfill the purpose of using the marked strain to assess its shedding pattern in animal feces, where it would be challenged by competition from indigenous microflora as well as other potentially stressful conditions, the fitness of the construct should match the growth behavior of the host strain and not be affected by the introduction of marker systems. Based on the result that no significant growth difference was detected between the wild type and its lux-construct in liquid medium, it is assumed that the expression of *luxCDABE* operon and the production of luciferase and its substrates did not add much metabolic burden on the host strain. Similar low fitness costs were also reported for similar chromosomal bioluminescence markers in *Salmonella* Typhimurium (Flentie, Qi et al. 2008) and *Pseudomonas fluorescens* (Amin-Hanjani, Meikle, 1993). The introduction of plasmid-borne markers, on the other hand, may affect the fitness of the construct, partly due to the increased expression of the higher copy of marker genes (Amin-Hanjani, Meikle, 1993). However, such was not the case in the plasmid-borne GFP marker constructed in this study.

Stability is another important criterion for the evaluation of a reliable marker system. The use of plasmid-borne bioluminescence markers was suggested, requiring additional selection pressure to maintain the plasmid in long

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term studies (Ritchie, Campbell et al. 2003), otherwise, as showed in one such construction in *Pseudomonas fluorescens* (Amin-Hanjani, Meikle, 1993), the lux-marker may have a half-life as short as 11.5 passages during subculturing in liquid media. In contrast, the chromosomally-integrated *luxCDABE* marker constructed by mini-Tn5 transposon mutagenesis in this study showed high stability in the host strain both *in vitro* and cattle shedding experiment. Although the loss of bioluminescence was observed once during the subculturing in a liquid medium, probably due to deletion or excision of the transposon, such was not noted during the two-week shedding experiment in cattle.

Compared to bioluminescent markers, the cell light output by GFP markers is more closely dependent on the copy number of the gfp gene; therefore, plasmid-bearing GFP markers were frequently used to track the survival of marked organisms in different environmental samples (Himathongkham, Bahari et al. 1999; Burnett, Chen et al. 2000; Gagliardi and Karns 2002; Wachtel, Whitehand et al. 2002; Franz, van Diepeningen et al. 2005). No loss of GFP marker was reported in these studies and, in one experiment (Franz, van Diepeningen et al. 2005), STEC O157:H7 containing a pGFP vector (a plasmid coding GFP) was detected for at most 133 days in cattle manure. In the current study, the same pGFP vector was used to mark O91:H21 strain, however, the marker showed poor stability both *in vitro* and in the cattle shedding experiment. Therefore, the plasmid-bearing GFP marker is not

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recommended for long term studies, unless antibiotic selection can be incorporated into the research methods.

During the shedding experiment, the discrimination using bioluminescence marker greatly complemented the inadequate selection of antibiotics. Interfering indigenous bacteria with similar resistance pattern (nalidixic acid and kanamycin resistance) was recovered on antibiotic-supplemented LB plates most of the time during the shedding experiment, but the use of bioluminescence marker could easily separate them in the dark. And according to the post-experiment M-PCR analysis, the discrimination was accurate and the enumeration results were reliable. Meanwhile, the enumeration of bioluminescent colonies only required an imaging system that can take pictures in the dark with extended exposure time. Such imaging systems can be easily found in most of the molecular biology laboratories.

One potential benefit of using bioluminescent markers is the estimation of vital cell concentration via the measurement of light intensity. Chromosome-bearing bioluminescent markers have the advantage of reflecting the number of light-emitting cells with high accuracy (Amin-Hanjani, Meikle, 1993). Therefore, there's a potential to develop a non-extractive and high-throughput enumeration method for STEC shedding experiments. However, compared to plasmid-bearing markers, the chromosome-bearing lux-markers are limited in low copy number of *lux* genes in one cell and, therefore, have higher minimum detection

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concentration of the organisms (Siragusa, Nawotka et al. 1999; Ritchie, Campbell et al. 2003). Previous studies have reported that minimums of 1.5 x 10^4 CFU/g of chromosomal lux-marked O157:H7 in soil (Ritchie, Campbell et al. 2003) and 4.2 x 10^3 to 8.0 x 10^4 CFU/ml of chromosomal lux-marked *Salmonella enterica* in clear medium (Howe, Karsi et al. 2010) were required for the detection of luminescence using luminometry. Later in this study, the minimum detection concentration of our lux-construct was also measured according to the method described previously (Howe, Karsi et al. 2010) and a minimum of approximately 4.5 x 10^4 CFU per gram feces was needed. Such detection limit could not fulfill the STEC shedding study in cattle as concentrations of the organism in fecal samples collected during most of the sampling points were below or around the minimum detection level. Further studies are needed. The construction of a chromosomal *luxCDABE* marker with multi-insertion or the development of a strong promoter for the expression of *lux* genes could increase detection sensitivity and may be considered for future improvement of this marker system.

To our knowledge, our study was the first study to report the shedding pattern of STEC O91:H21 in cattle feces. Compared to the fecal shedding pattern of O157:H7 by calves receiving the same dose, the shedding magnitude of our O91:H21 strain was relatively lower: 1.2 x 10^3 to 1.8 x 10^4 CFU/g in O91:H21 shedding compared to 7.9 x 10^3 to 4 x 10^6 CFU/g in O157:H7 shedding at day 2 post inoculation (Brown, Harmon et al. 1997); meanwhile the highest shedding

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magnitude of O157:H7 by individual calves could reach as high as approximately 10^8-10^9 CFU/g (Cray and Moon 1995), such high levels were not observed here and the highest value in this study was 9.3 x 10^4 CFU/g. The explanation for the difference of shedding levels between these two serotypes was not clear. One possible explanation is the variety in fitness of two strains when in the presence of indigenous intestinal microflora. Additionally, because of the different diets, animal sources, husbandry methods, etc., some intestinal physiological conditions impacting bacteria growth and proliferation could differ between the calves used in different experiments (Widiasih, Ido et al. 2004). A greater shedding magnitude of O157:H7 suggests a higher chance to contact the organism; however this doesn't make the shedding of O91:H21 less of concern. A previous survival study (Molina, Parma et al. 2003) in acidic and alcoholic condition indicated that O91:H21 may be more resistant to stress conditions than other STECs, including O157:H7. Therefore, this strain may persist within the environment or contaminated food for a longer time. As for the shedding duration, because the shedding experiment in this study only lasted for two weeks, it was not known whether the fecal shedding of O91:H21 by calves could last for as long as 20 weeks as observed in an O157:H7 shedding experiment (Cray and Moon 1995). Calves in this study that were tested negative of the organism at day 12 or day 14 PI may still carry the organism in their intestine and could shed the organism intermittently if the experiment was extended.

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The contents of the cecum and colon tested positive for the O91:H21 in all animals at day 14 PI, despite the fact that fecal samples from two of the animals tested negative for the organism. This is consistent with many other studies that considered the large intestine as the main habitat for STEC in cattle (Grauke, Kudva et al. 2002).

Based on the results of this study, it is suggested that a chromosomal based *luxCDABE* marker may be a better model than antibiotic resistance markers for the study of STEC localization and shedding in cattle.

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APPENDIX

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$$Y = N_0 + A \times exp\left\{-exp\left[\frac{\mu_{max} \times e}{A}(\lambda - x) + 1\right]\right\}$$

Figure 1. *Gompertz* equation for the modeling of bacterial growth curve. Originally in the equation, Y is the natural logarithm value of cell number, N_0 is the natural logarithm value of initial cell number, A is the difference between initial and final cell numbers, μ_{max} is the maximum growth rate and λ is lag time. Because there's a linear relationship between the OD_{600} value and cell number, OD_{600} values were interpolated where cell numbers were in the equation.

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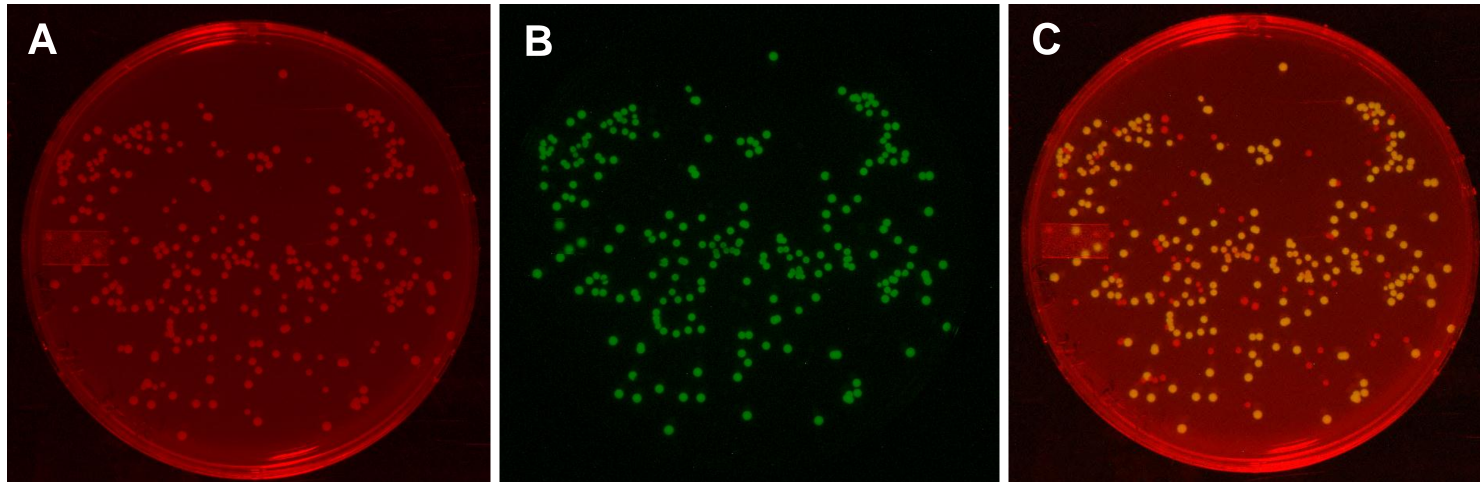

Figure 2. Differentiation of bioluminescence-positive colonies from negative colonies on LB agar containing 50 ug/ml nalidixic acid and 50 ug/ml kanamycin using the FluorChem® imaging system. (A) the image of a plate that was taken under artificial white light displayed both bioluminescence-positive and negative colonies that were growing on the plate; (B) the image of the same plate that was taken in the dark displayed only the bioluminescence-positive colonies; (C) for a convenient separation of bioluminescence-positive colonies from negative colonies on a cluttered plate, the Overlay function in AlphaEase® FC software (Alpha Innotech Corp, San Leandro, CA) was used to mark two images with different colors (red and green in this example) and overlay one image on the other. Bioluminescence-positive colonies appeared yellow, and bioluminescence-negative colonies appeared as the original red color.

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Figure 3. API 20E test (BioMerieux Vitch, Inc., Hazelwood, MO) for *luxCDABE*-marked O91:H21 construct and its donor and recipient strains.

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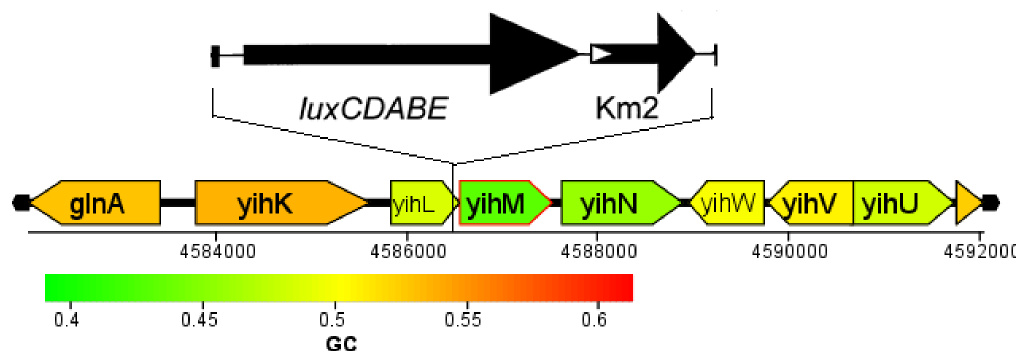
(A)



(B)

1	GCCTGGTTTCATATGAATATCAAGGTTCAGACGGCGGGCTTTATCTCCAAAGGCAGAGAG
61	TTCTTTATCGTCCATATTACCAAGAGAAAAACGCTCGCCATCCAGAACATGGATTTTGAC
121	GCCACGTAGATTATTTTCACTTGCGATATCAAGTAAATCAGCAGGCAACACTCTTTCCAG
181	CCGCATATTTAAATGAAAGGCATAAGCATGTAAATAAAGAGGAAGAGTGTCGACACGTTG
241	TAGAATCTTTCTTGCGTTATTTATTGTCACCATAAAACACCTACAGATTAATGATGAGGT
301	CTACGG[CTGTCTCTTGATCAGATCT]GGCCGCCTAGGCCGAATTCGAGCTCGGTACCCGG
361	GGA

Figure 4. The insertion site of *luxCDABE*-carrying mini-Tn5 transposon on chromosome (A) the insertion site was 27bp before the stop codon of *yihL* gene, according to the complete genome sequence of *Escherichia coli* CFT073. (B) DNA sequence of PCR product amplified by primers luxE and Yih. The box indicates the position of the I-end of mini-Tn5 transposon.

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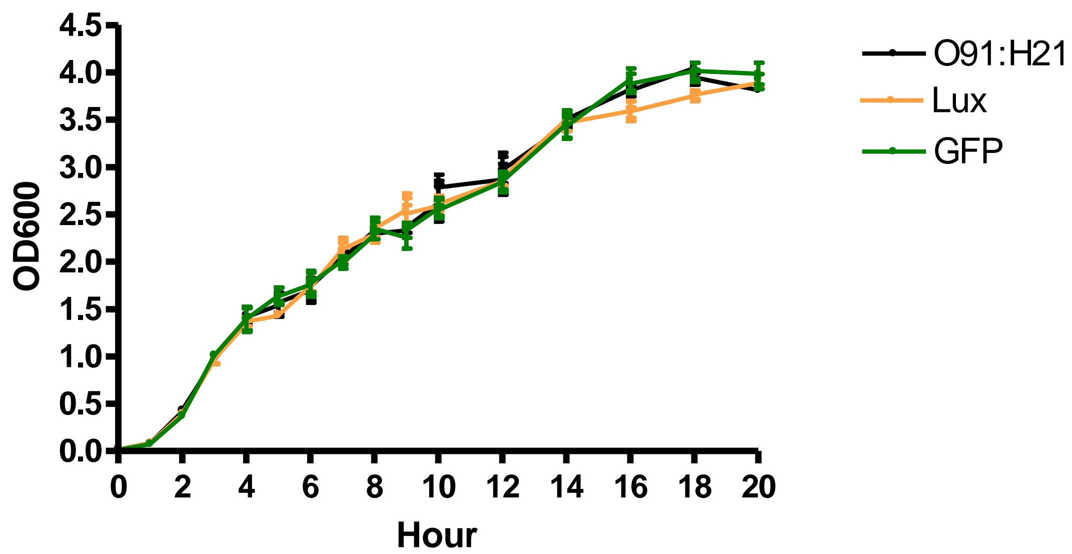

Figure 5. Growth curves of O91:H21 and its Lux- and GFP-marked constructs in LB broth

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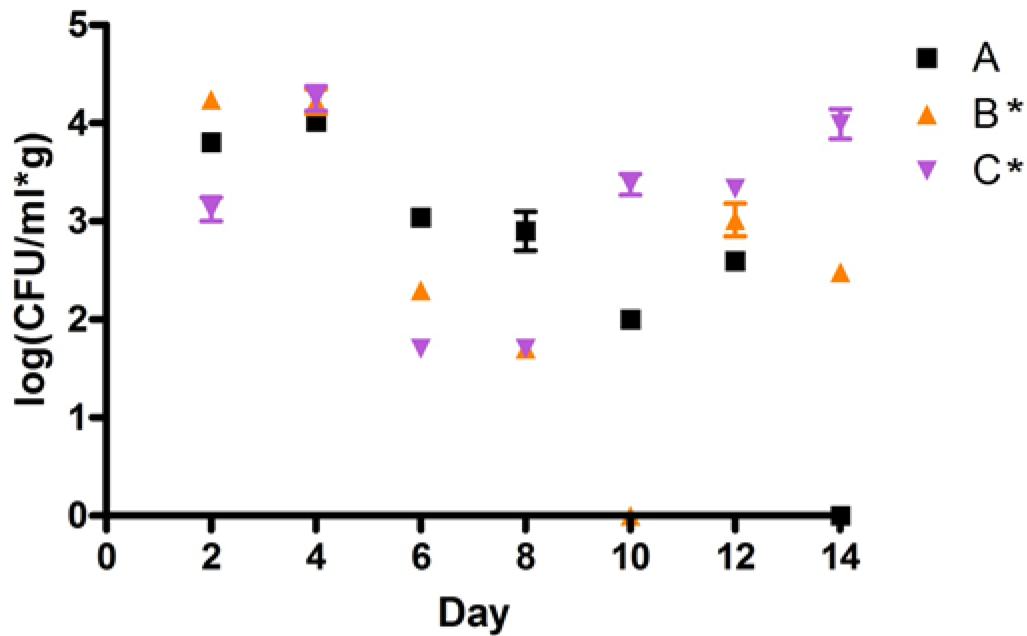

Figure 6. Fecal shedding levels of wild type O91:H21 in three calves with experimental infection

*Indigenous bacteria exhibiting nalidixic acid resistance was found among colonies recovered from fecal samples collected at day 12 and day 14 PI in calf B and from samples collected from day 10 to 14 PI from calf C. Therefore, the fecal shedding levels at these sampling points in calf B and C were not reliable.

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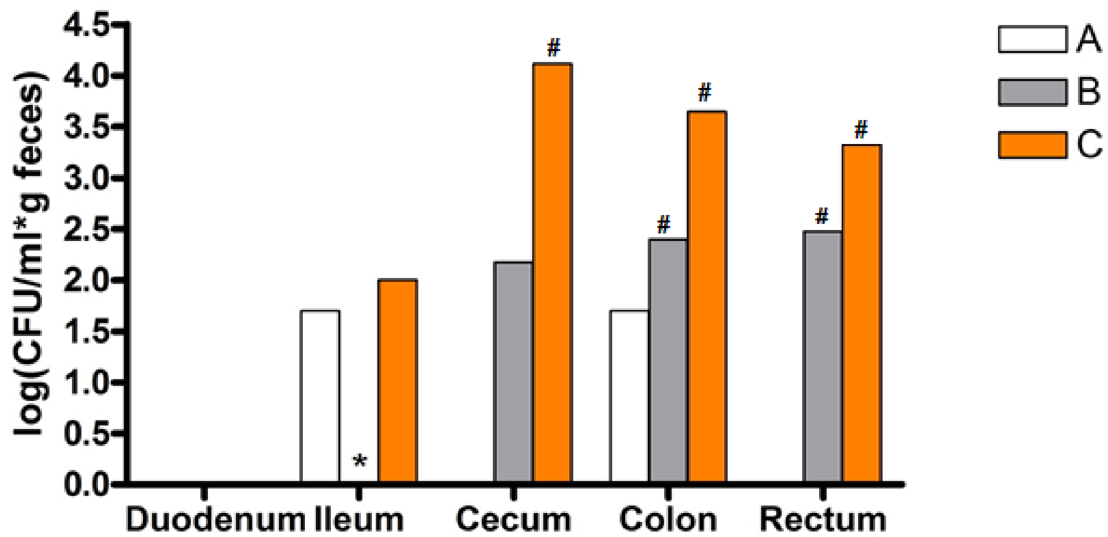

Figure 7. Concentration of the wild type O91:H21 in bovine intestinal contents at day 14 PI.

*Insufficient quantity of ileal contents was available for this calf during the sampling

#Indigenous bacteria exhibiting nalidixic acid resistance was found among colonies recovered from fecal and intestinal samples collected from calf B and C and was observed to confound the enumeration results. Therefore, the concentrations were not reliable.

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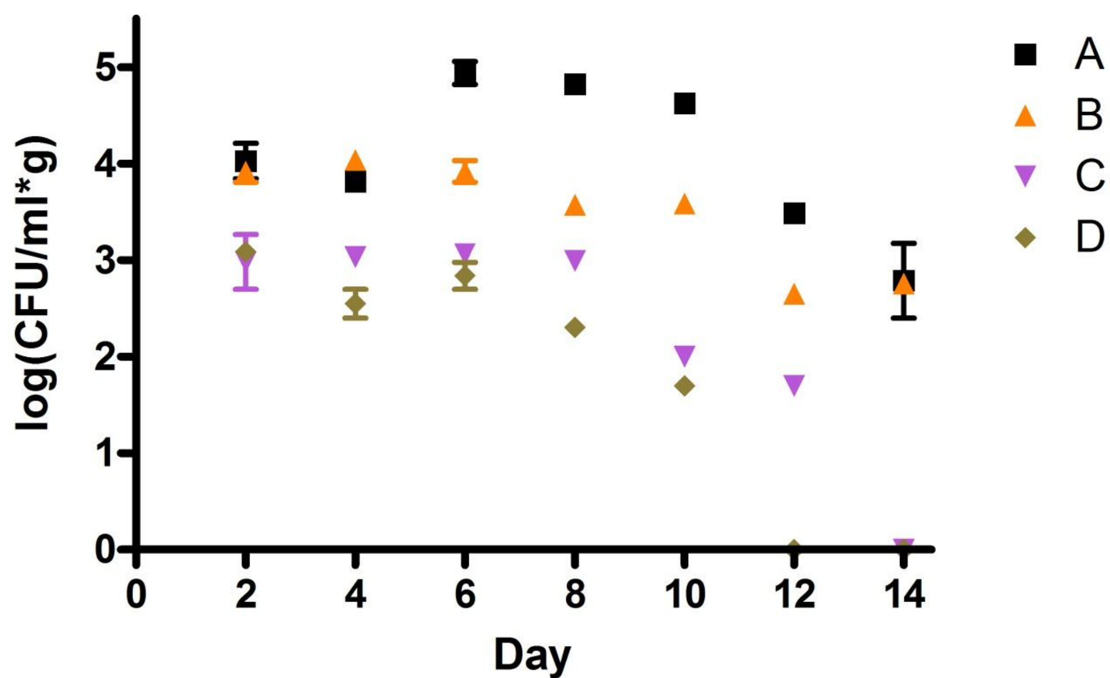

Figure 8. Fecal shedding levels of *luxCDABE*-marked O91:H21 in four calves with experimental infection

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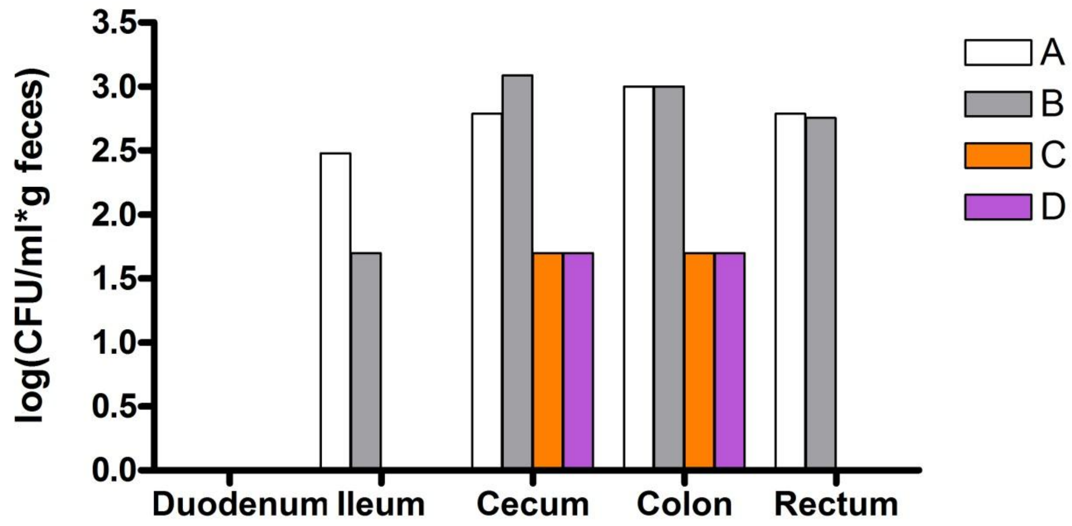

Figure 9. Concentration of the *luxCDABE*-marked O91:H21 in bovine intestinal contents at day 14 PI.

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VITA

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