Identifying the Shedding Patterns of Wildlife *Escherichia coli* O157:H7 in Experimentally Inoculated Neonatal Jersey Calves

Xunde Li1,2, Elizabeth M Antaki Zukoski1,2, Tran HB Nguyen1, Bruce R Hoar3, Michele T Jay Russell2,4, and Edward R Atwill1,2*

1Department of Population Health and Reproduction, University of California, USA
2Western Institute for Food Safety and Security, University of California, USA
3College of Agriculture and Natural Resources, University of Wyoming, University, USA
4Western Center for Food Safety, University of California, USA

**Abstract**

Cattle are considered to be a major reservoir host of Shiga-toxin producing enterohaemorrhagic *Escherichia coli* O157:H7. Contaminated foods of bovine origin that have been exposed to bovine or wildlife *E. coli* O157:H7 isolates are important vehicles of human infections, which can lead to the deadly hemolytic uremic syndrome (HUS). The objective of this study was to determine possible dose-dependent pathogen shedding patterns of a wildlife *E. coli* O157:H7 strain in neonatal calves. We inoculated six calves, 1-2 day old calves, with either 10^4 or 10^5 CFU and collected fecal samples for 11 days post-inoculation to determine the shedding patterns of the inoculated *E. coli* O157:H7. Calves were then euthanized, with tissues collected for culture. One calf inoculated at 10^5 CFU and both calves inoculated at 10^4 CFU became infected. The calf inoculated at 10^5 CFU, which shed the *E. coli* O157:H7 isolate for the entire 11 days post-inoculation, had a wider distribution of colonized tissues compared to the other 10^4 CFU and 10^5 CFU inoculated calves. We conclude that the wildlife strain has a dose-dependent shedding pattern with a calculated infectious dose (ID50) of 10^5 CFU.

**ABBREVIATIONS**

- EHEC: Enterohaemorrhagic *Escherichia coli*; *E. coli* O157:H7: *Escherichia coli* O157:H7; Stxs: Shiga toxins; HUS: Hemolytic Uremic Syndrome; A/E: Attaching/Effacing; ID50: 50% Infectious Dose; BHI: Brain Heat Infusion; PBS: Phosphate Buffered Saline; LB: Luria Base; eaeA: Intimin; HlyA: Hemolysin A; IACUC: Institutional Animal Care and Use Committee; TRACS: Teaching and Research Animal Care Services; RAMS: Rectoanal Mucosal Swabs; TSB: Tryptic Soy Broth; IMS: Immunomagnetic Separation; PFGE: Pulsed Field Gel Electrophoresis

**INTRODUCTION**

*Escherichia coli* are a Gram-negative, rod-shaped bacterium of the Enterobacteriaceae family. Most *E. coli* strains colonize the gastrointestinal tract of humans and mammals as part of the normal flora. However, some can evolve into pathogenic strains by acquiring virulence factors through plasmids, transposons, bacteriophages, and/or pathogenicity islands. These pathogenic *E. coli* are characterized by serogroups, pathogenicity mechanisms, clinical symptoms, or virulence factors [1,2]. One major pathogenic group of *E. coli* strains are the enterohaemorrhagic *Escherichia coli* (EHEC), which can produce Shiga toxins (Stxs) and cause non-specific diarrhea, hemorrhagic colitis, and potentially life-threatening hemolytic uremic syndrome (HUS) in humans. The occurrence of massive outbreaks of Shiga toxin-producing *E. coli* infections, especially resulting from the serotype O157:H7 and the risk of developing HUS (the primary cause of acute kidney failure in children in many developed countries) make this pathogen a public health problem of serious concern [3]. Sources of human infection include contaminated foods, primarily ground beef, raw milk, lettuce, spinach, and fresh fruits, as well as waterborne transmission in rural areas through contact with feces from infected animals [4,5].

The natural reservoir of *E. coli* O157:H7 is in the intestinal tracts of animals, particularly cattle and small domesticated ruminants, like sheep and possibly goats. It can also be carried by pigs, poultry, other domestic farm animals, birds, insects, and wildlife, such as rodents and white-tailed deer. Prevalence within wildlife carriers can be low, but can provide a resource for transmission within local areas and distant geographic locations [6-8]. Ferens and Hovde, 2011, found *E. coli* O157:H7 in 11.4% of cattle (n=1407), 1.2% of swine (n=1102), 3.6% of sheep and goats (n=364), and in 5.2% of 154 fly pools, where some isolates from cattle, swine, and flies shared indistinguishable subtypes of the bacteria. In another study, 1,071 wild rodents were trapped from October 2009 to August 2011 in the Salinas Valley region to...
determine the fecal prevalence and risk factors associated with the occurrence of *E. coli* 0157:H7, *Salmonella* spp., *Cryptosporidium* spp., and *Giardia* spp. within agricultural production systems. It was determined that 0.2% of the fecal samples collected from the wild rodents were positive for *E. coli* 0157:H7 [9].

There has been extensive work using animal models to better understand the dynamic pathogenic mechanisms of EHEC, especially with regards to serotype 0157:H7. It has been shown that neonatal animals, especially calves, are the susceptible age class for these types of diseases and symptoms, which include diarrhea, hyperemia, fibrinous exudates, enteritis, and the formation of attaching/effacing (A/E) lesions within the intestinal tissues. In contrast, older ruminants have transient watery diarrhea, but are not seriously affected by an *E. coli* 0157:H7 infection. It is thought that *E. coli* 0157:H7 primarily colonizes the rectoanal junction in cattle, which is then responsible for the persistent shedding pattern. The capacity for persistence with episodic shedding has been seen in studies, but there also can be a significant detection problem since the infected animals can be asymptomatic [10-13].

To reduce the risk of *E. coli* 0157:H7 transmission from animals to humans, it’s necessary to determine if the bacterium’s pathogenesis differs among various wildlife strains. Here, we examined the shedding patterns among neonatal jersey calves inoculated with a wildlife *E. coli* 0157:H7 strain isolated from an agricultural production system. Our objective was to determine dose-dependent characteristics in the host clinical response correlated with isolation in daily fecal excretion and tissue-based cultures.

**MATERIALS AND METHODS**

**Standard growth curve of bacterial strain**

The wildlife *Escherichia coli* 0157:H7 strain was isolated from a deer mouse (*Peromyscus maniculatus*) November 2009 in Monterey County, CA on a spinach produce ranch. Three growth curves were made by incubating a bead of stock inoculum, taken from -80°C frozen banks (Pro-lab, Richmond Hill, Ohio, USA), in 150mL of brain heart infusion, BHI (Sigma-Aldrich, St. Louis, Missouri, USA), stored at -80°C. Each broth was incubated, plated, and original concentrations were calculated as previously described by Antaki-Zukoski et al., 2018 [14]. The *E. coli* 0157:H7 strain was also evaluated for both Shiga toxins, intimin (eaeA), and hemolysin A (HlyA) virulence genes using an endpoint analysis (Eppendorf, Hauppauge, New York, USA) according to the PCR protocol [3] (data not shown).

**Ethics statement**

All animal experiments were conducted under the approval by the Institutional Animal Care and Use Committee (IACUC) at the University of California Davis Animal Care and Use program (AUP #15459). Experimental procedures followed the federal guidelines outlined in the “Animal Welfare Act” and “Health Research Extension Act”, where personal protective equipment, standard operating protocols, daily cleaning/observation/animal enrichment, and sedation/euthanasia necropsy methods were described in detail for the review and approval by the committee.

**Animal experiments**

Six male calves 1-2-days-old that had received colostrum were purchased from an approved dairy. Each calf was housed individually in the biosafety level-II animal facility at the Teaching and Research Animal Care Services (TRACS). The calves were fed commercial milk replacer without antibiotics twice a day and given free access to water. Bedding material was removed while pens were bleached and rinsed daily, to ensure fresh fecal collection. Upon arrival, each calf was deemed healthy by clinical examination and hematological analysis with a serum chemistry panel and complete blood count. Additionally, each calf was tested for the presence of *Cryptosporidium* spp. with an acid-fast stain performed on feces. In order to confirm that calves were not colonized with *E. coli* 0157:H7, fresh rectal fecal samples and rectoanal mucosal swabs (RAMS) were collected for three consecutive days prior to inoculation with the wildlife *E. coli* 0157:H7 [15]. Ten grams of each fecal sample was measured into 90mL of Tryptic Soy Broth (TSB) (Sigma-Aldrich, St. Louis, Missouri, USA), while the RAMS were placed in 50mL of TSB. The samples were incubated at 25°C for 2 hours, 42°C for 8 hours followed by immunomagnetic separation (IMS) using anti-O157 antibodies (Dynal Inc, Camarillo, California, USA) and cultured on MacConkey agar with sorbitol, cefixime, and potassium tellurite (Becton, Dickinson, Co, Sparks, Maryland, USA), and Rainbow agar (Biolog, Hayward, California, USA) for the presence of *E. coli* 0157:H7 [14]. If any colonies were considered *E. coli* 0157 suspects, traditional PCR was performed using a set of specific primers to detect O-antigen-encoding rib regions of *E. coli* 0157:H7 [3]. Once the calves were confirmed negative for *Cryptosporidium* spp. and *E. coli* 0157:H7 prior infections, they were placed in individual pens, separated by one pen length. They were then assigned an *E. coli* 0157:H7 inoculum dose.

**Inoculation of animals**

One micro bank bead for the wildlife strain, stored at -80°C, was placed in a 250mL flask containing 150mL of BHI. The flasks were incubated at 37°C for 6 hours, while shaking at 100 rpm. Once the determined optical density was reached, the flasks were placed on ice for 15 minutes. At this point the optical density was measured again and the concentration of the stock solution was calculated by using the growth curve from the *E. coli* 0157:H7 strain. From the known stock solution concentration, the inoculums were prepared for the wildlife *E. coli* 0157:H7 strain in 200mL of PBS.

In previous lab studies inoculating neonatal calves with wildlife *E. coli* 0157:H7 strain isolated from a feral swine, at 10⁴ CFU and 10⁵ CFU showed that these inoculum doses were not high enough to cause an infection (data below). To ensure the feral swine and deer mouse strains were distinct from one another and from other *E. coli* 0157:H7 isolates collected in California, a Pulsed Field Gel Electrophoresis (PFGE) was performed. The isolates were digested using the Center for Disease Control and Prevention PulseNet PFGE with XbaI restriction enzyme [16]. A dendogram was constructed by analyzing each digest profile with band matching and phylogenetic clustering analysis methods in Gel ComparII (Applied Maths, Sint-Martens-Latem, Belgium) computer software. For this study, assuming that the two wildlife *E. coli* 0157:H7 strains have similar pathogenicity patterns once
inoculated into a host, one calf served as a negative control, two calves were inoculated with $10^8$ CFU (calf A and calf B), two calves were inoculated with $10^9$ CFU (calf A and calf B), and one calf was inoculated with $10^{10}$ CFU (positive control) of the deer mouse wildlife E. coli O157:H7 strain. Serial dilutions from $10^4$ to $10^{10}$ were made in 9ML of PBS and plated in triplicate on LB for each stock solution and inoculum. After an overnight incubation at 37°C, the true concentration of each inoculum was calculated. The individually housed calves were intragastrically inoculated with their assigned inoculum dose in 200 ml PBS with a calf gastric feeder (VetOne, Boise, Idaho, USA) followed with another 200mL of PBS as a wash.

**Daily sampling and fecal scoring**

Fecal samples and RAMS were collected twice daily for the first two days post-inoculation (p.i.) and then collected once a day until the 11th day p.i. Fecal consistency was scored on a scale from 1-4, with 1=Normal, 2=Paste, 3=Liquid, and 4=Water (Table 1). All of the samples were collected in the afternoon and transported back to the laboratory in a refrigerated state. The samples were processed within 24 hours of sampling.

**Analytical assays for detecting E. coli O157:H7**

Each 10g fecal sample and RAMS were plated in separate whirl packs (eNasco, Fort Atkinson, Wisconsin, USA) containing either 90mL TSB for fecal and 50mL TSB for RAMS, and then incubated for 2 hours at 25°C, 8 hours at 42°C, and held overnight at 6°C. E. coli O157:H7 was recovered using IMS and the Dynal Bead Retriever, with 50 μL of washed beads streaked for isolation on Rainbow agar and another 50 μL streaked for isolation on Sorbitol MacConkey Agar [14]. As performed in our laboratory, this IMS method has been shown to be able to detect as few as 1CFU/10g of calf feces (Data not shown). Two suspect colonies per positive plate were confirmed as E. coli O157:H7 using PCR as described above [17]. Confirmed colonies were stored at -80°C in Microbank vials for further analysis.

**Euthanasia and necropsy**

All calves were euthanized with pentobarbital sodium (Euthasol solution, Virbac, Fort Worth, Texas, USA) intravenously and necropsied on the 11th day p.i. Intestinal sections of the duodenum, jejunum, ileum, cecum, spiral colon, distal colon, and rectum were collected for bacterial culture (approximately 5cm in length). Sections of pancreas, liver, mesenteric lymph node, gall bladder, lung and kidney were also taken for culture (approximately 5g). Each segment of intestine was tied with string by both ends so that the contents and the mucosa could be cultured for the presence of E. coli O157:H7. Once transported to the laboratory, one end of the tissue segment was cut and the contents were collected in a whirl pack bag containing 90mL of TSB. The intestinal tissue was then cut longitudinally to expose the mucosa and placed in a separate whirl pack bag with 90mL of TSB. The non-intestinal tissues were each placed in a bag containing 50mL of TSB. All TSB bags were then incubated for 2h at 25°C, 8h at 42°C, and then held overnight at 6°C. E. coli O157:H7 was recovered using the same method as described above.

**Data analysis**

The ID50 was determined for the wildlife E. coli O157:H7 strain with binary logistic regression using StataCorp LP, College Station, Texas, USA) computer software by calculating the number of calves that became infected over the total number of calves used for each inoculum dose. With these percentages, a curve was generated to determine the dose at which 50% of the calves became infected with the inoculated E. coli O157:H7 strain.

**RESULTS AND DISCUSSION**

Our definition of an infected calf was one that shed the wildlife E. coli O157:H7 inoculum for more than 48 hours p.i. and was culture positive in at least one of its tissues at necropsy on day 11 p.i. This was to account for passage of the inoculum through the gastrointestinal tract without attaching/colonizing the actual tissues. The wildlife E. coli O157:H7 strain used for this study was PCR-confirmed positive for both Shiga toxins, the attaching/facing (eaeA), and the hemolysin A (HlyA) genes. By PFGE analysis, the deer mouse E. coli O157:H7 strain was different from the feral swine strain as well as three other E. coli O157:H7 strains collected from bovine feces in various parts of California (Figure 1).

Higher average fecal scores were observed in the $10^8$ CFU inoculum group compared to the calves inoculated with $10^9$ CFU. The positive control calf stayed consistent at a score of 1, while the negative control calf fluctuated between a score of 1-3 (Figure 2). IMS and culture was performed on daily fecal and RAMS collections for the detection of the inoculated E. coli O157:H7 strain. Calf B from the low inoculum dose shed for six days, while Calf A stopped shedding after four days post-inoculation. Both calves inoculated at 109 CFU shed longer than the lower dose inoculum group, with Calf B shedding for 7 days and the Calf A shedding for the entire duration of the trial. The positive control also shed the entire experimental time of eleven days. The E. coli O157:H7 inoculum was never recovered from the negative control. Table 2 represents the IMS daily collection results.

Since the negative control calf received fecal scores between 1-3, it was determined that there could be another pathogen causing these clinical signs. A possible conclusion was that the calf contracted Cryptosporidium spp. before the experiment and was pre-screened negative, but the infection caused clinical signs while in the experiment. It has been reported that neonatal calves infected with the protozoan Cryptosporidium spp. are usually asymptomatic, and mainly stressed animals show clinical manifestations of the disease associated with various degrees of diarrhea [18]. The negative control calf might have been exposed to the Cryptosporidium spp. protozoa prior to or during transportation, and then stressed due to the experiment,
Figure 1 PFGE dendogram of both wildlife and bovine Escherichia coli O157:H7 isolated from various parts of California, USA. Bovine strain 8055 isolated from Yolo County, Deer mouse inoculum strain isolated from Monterey County, Bovine strain 14A isolated from Imperial County, Bovine strain W1 isolated from Colusa County, and Feral pig strain 1174 isolated from San Benito County.

Figure 2 Average daily fecal scores for each inoculum group of experimental calves. Negative control: negative control calf; Lower inoculum group: average fecal scores from the calves inoculated with $10^8$ CFU of the wildlife Escherichia coli O157:H7; Higher inoculum group: average fecal scores from the calves inoculated with $10^9$ CFU of the wildlife Escherichia coli O157:H7; Positive control: positive control calf inoculated with $10^{10}$ CFU of the wildlife Escherichia coli O157:H7.

Table 2: Daily collection IMS results for shedding of the wildlife Escherichia coli O157:H7 in each calf following oral inoculation.

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>Wildlife E. coli O157:H7 isolated from a Feral Swine</th>
<th>Wildlife E. coli O157:H7 isolated from a Deer Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative Calf A $10^6$CFU</td>
<td>Calf B $10^6$CFU</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

(+) indicates presence of the Escherichia coli O157:H7 inoculum tested by Immunomagnetic Separation and PCR-confirmed on the day of collection from each calf; (-) indicates the Escherichia coli O157:H7 inoculum was not detected on the day of collection by Immunomagnetic Separation from each calf; (N/A) calves were euthanized the day prior.
causing clinical signs of diarrhea. This is in comparison to the positive control that had normal feces throughout the entire experiment. Consequently, clinical signs of diarrhea and fecal score differences between in oculum doses could not be correlated to the \( E.\ coli \) \( O157:H7 \) inoculum.

Due to animal welfare, a low dose inoculum experiment was not performed with this wildlife \( E.\ coli \) \( O157:H7 \) strain, but an \( E.\ coli \) \( O157:H7 \) strain isolated from feral swine was experimentally inoculated in neonatal calves in previous studies using doses of \( 10^8 \) CFU and \( 10^9 \) CFU (Table 2). Since no animals were considered to be infected with the feral swine strain, higher inoculum doses were used instead for the current study. This is a limitation, but assuming the two strains behave the same in neonatal calves, the infectious dose at 50% was calculated to show that \( 10^8 \) CFU is the ID50 value for the deer mouse wildlife \( E.\ coli \) \( O157:H7 \) strain.

On the 11th day post-inoculation, the calves were euthanized and necropsied. IMS and culture were performed on all tissues and intestinal contents that were collected from each calf. The wildlife \( E.\ coli \) \( O157:H7 \) inoculum was never recovered from the pancreas, liver, mesenteric lymph node, gall bladder, lung, or kidney from any of the calves. Recovery of \( E.\ coli \) \( O157:H7 \) wildlife strain from tissues is shown in Table 3. The \( E.\ coli \) \( O157:H7 \) inoculum was recovered from at least one intestinal tissue in all calves except for the negative control and Calf A inoculated at \( 10^8 \) CFU. Due to its negative fecal and RAMS cultures after four days post-inoculation and tissue culture at necropsy, by our case definition Calf A at \( 10^8 \) CFU was considered not infected.

The infectious ID50 for the wildlife \( E.\ coli \) \( O157:H7 \) strain was calculated to be \( 10^8 \) CFU. One of two calves inoculated at \( 10^8 \) CFU was considered infected. Both calves inoculated at \( 10^9 \) CFU were deemed infected due to their long shedding period of \( E.\ coli \) \( O157:H7 \) [2,19]. Although the case numbers are limited, there was also a dose-dependent trend seen in the cultured tissues that were taken from each calf. It appeared that if a calf was inoculated with the higher dose of the wildlife \( E.\ coli \) \( O157:H7 \), it would shed for a longer period of time and have culture-positive tissues when tested at the end of the experiment.

### CONCLUSION

Wild animals can potentially carry \( E.\ coli \) \( O157:H7 \), causing transmission to humans when they are in contact with farm animals or produce fields [20]. Only a limited number of experimental studies have evaluated the ability of wildlife species carrying and transmitting the \( E.\ coli \) \( O157:H7 \) pathogen through the environment or to other animals. Due to this limitation, we don’t fully understand the potential virulence of these wildlife isolates. We have shown in this study that a wildlife \( E.\ coli \) \( O157:H7 \) isolate, from a deer mouse, has the capability to experimentally infect neonatal calves at a high inoculum dose of \( 10^8 \)CFU, with a wide range of intestinal tissue colonization if infection continues for a week or longer. Future studies could further examine the host-pathogen interaction between these wildlife isolates and their reservoirs by determining the infectious dose/clinical signs in deer mouse models.

### ACKNOWLEDGEMENT

The authors are grateful to the Teaching and Research Animal Care services for their assistance on the purchasing, care, and transportation of the animals used for this experiment. The authors would also like to thank the California Animal Health and Food Safety, University of California, Davis, for their necropsy floor and their overall assistance. Support for this research was provided to the Western Center for Food Safety contract U01-003-572 from the U.S. Food and Drug Administration. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the U.S. Food and Drug Administration.

### REFERENCES

9. Kilono C, Li X, Vivas EJ, Jay-Russell MT, Fernandez KL, Atwill ER. Fecal shedding of zoonotic food-borne pathogens by wild rodents in a...


Cite this article