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### Detection and source tracking of Escherichia coli, harboring intimin and Shiga toxin genes, isolated from the Little Bighorn River, Montana

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## Detection and source tracking of *Escherichia coli*, harboring intimin and Shiga toxin genes, isolated from the Little Bighorn River, Montana

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The Little Bighorn River flows through the Crow Indian Reservation in Montana. In 2008, *Escherichia coli* concentrations as high as 7179 MPN/100 ml were detected in the river at the Crow Agency Water Treatment Plant intake site. During 2008, 2009, and 2012, 10 different serotypes of *E. coli*, including O157:H7, harboring both intimin and Shiga toxin genes were isolated from a popular swim site of the Little Bighorn River in Crow Agency. As part of a microbial source tracking study, *E. coli* strains were isolated from river samples as well as from manure collected from a large cattle feeding operation in the upper Little Bighorn River watershed; 23% of 167 isolates of *E. coli* obtained from the manure tested positive for the intimin gene. Among these manure isolates, 19 were identified as O156:H8, matching the serotype of an isolate collected from a river sampling site close to the cattle feeding area.

**Keywords:** sewage pollution; river water; water pollutants; water-borne diseases

### Introduction

The Little Bighorn River flows through the heart of the Crow Indian Reservation in southeast Montana. With its headwaters beginning in Wyoming just south of the Montana border, the river flows north through the three towns of Wyola, Lodge Grass, and Crow Agency. Members of the Crow tribal community depend on the Little Bighorn River for a variety of purposes. During summer and autumn months, the river is used recreationally by children for swimming and by adults for sport and subsistence fishing. Year round, the river provides drinking water for livestock. The primary source of water for residents in the many homes located close to the banks of the river is often a shallow well that may be hydraulically linked to the river. The Little Bighorn also plays an important role in the spiritual life of the Crow people. The river is used for traditional bathing after sweat lodge ceremonies, and as the source for pouring and drinking water during ceremonies.

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In recent years, the local tribal community has voiced concerns over degradation of water quality in the Little Bighorn and its tributary streams, with mention of warmer water temperatures, higher turbidity, and decreasing fish and freshwater mussel populations (Cummins, Doyle, Kindness, Lefthand, et al. 2010). In light of the deteriorating water quality, tribal members created the Crow Environmental Health Steering Committee. The Committee, in the framework of a community-based participatory research (CBPR) project, functions in a unique collaboration with staff and students of the local Little Big Horn College (LBHC) located in Crow Agency and Montana State University (MSU) – Bozeman, to conduct environmental health surveillance, to share information about water quality and environmental health issues with the community at large, and to formulate solutions to water quality issues.

The US Environmental Protection Agency (EPA) sets a guideline for bathing (swimming) recreational water use for *Escherichia coli* of no more than 126 CFU/100 ml, calculated as the geometric mean of no fewer than five sample readings over a 30-day period (USEPA 1986). Researchers from LBHC and MSU have measured high levels of *E. coli* in tribal rivers and streams that exceed this EPA guideline. As an example, in 2008, an *E. coli* concentration of 7179 MPN/100 ml was measured in a river sample collected at the Crow Agency Water Treatment Plant intake site (Cummins, Doyle, Kindness, Young, et al. 2010). Presence of *E. coli* in water is considered to be an indicator of fecal contamination (APHA 1998), and raises the possibility of health risks to humans posed by enteric pathogens present in fecal pollution (Wade et al. 2003). Based on a meta-analysis, Wade et al. (2003) concluded that presence of *E. coli* in fresh water (vs. marine waters) is “a more consistent predictor of gastrointestinal illness” than other bacterial indicators. In rural settings, water quality can be negatively impacted by fecal pollution from domestic sewage, and by ranching, agricultural, and pasture runoff. Failure to take appropriate preventive measures to protect water resources can contribute to poor water quality and increased public health risk (USEPA 2001). Since there are numerous homes and ranching operations located close to riverbanks along the entire length of the Little Bighorn River, there is concern that leaking septic systems and livestock may be potential sources of fecal contamination and high levels of *E. coli*.

For regulatory and public health agencies to address *E. coli* and fecal pollution of the Little Bighorn River, and for resource managers to institute best-management practices (Vogel et al. 2007), the sources of fecal contamination should be identified. Microbial source tracking (MST) methods (Simpson et al. 2002; Meays et al. 2004; USEPA 2005a; Field & Samadpour 2007) can be used to identify sources of *E. coli*. One type of MST strategy is referred to as library-dependent. These studies rely on creation of a reference library or database of molecular patterns (e.g. DNA fingerprints) derived from bacteria isolated from fecal material from known sources (Stoeckel & Harwood 2007). DNA fingerprints of bacteria isolated from water “down-stream” of the known sources of fecal bacteria are then compared to the source library’s database, using an appropriate method of analysis to check for similarity (Hassan et al. 2005). Repetitive element-based PCR (rep-PCR) is a widely used method of generating DNA fingerprints; rep-PCR uses various DNA probes to amplify sequences present in multiple copies in bacterial genomes and has been widely applied to MST studies (Versalovic et al. 1991; de Bruijn 1992; Vesalovic et al. 1994; Dombek et al. 2000; McLellan et al. 2003; Seurinck et al. 2003; Mohapatra et al. 2007).

Cattle can asymptotically harbor and shed micro-organisms that are pathogenic for humans, and are a primary reservoir for transmission of *E. coli* O157:H7 (Gyles 2007; Ferens & Hovde 2011). First described in a disease outbreak occurring in 1982

(Riley et al. 1983), *E. coli* O157:H7 is the prototypic form of the enterohemorrhagic *E. coli* (EHEC) pathotype capable of causing severe gastrointestinal disease in humans. Symptoms include bloody diarrhea and vomiting; the complication of hemolytic uremic syndrome (HUS) may lead to kidney failure and death (Tarr 1995). The more severe sequelae associated with O157:H7 infection are attributed to EHEC production of Shiga toxins, potent inhibitors of protein synthesis that cause apoptosis of infected cells within the kidney and other affected tissues (Donnenberg & Whittam 2001; Bolton 2011). Another key virulence determinant found in EHEC is intimin, an adhesin involved in intimate attachment of the bacteria to host intestinal tissue (Bolton 2011).

A study examining O157:H7 prevalence in range cattle herds from five states (including Montana) found that 13 of 15 herds sampled were positive for O157:H7, with up to 20% of animals within positive herds shedding O157:H7 bacteria in their feces (Laegreid et al. 1999). O157:H7 strains have been shown to be able to survive in bovine manure and associated pasture soils for several months, even with exposure to below freezing temperatures (Kudva et al. 1998; Bolton et al. 1999). In such a setting, contaminated soil may effectively act as a “vector and reservoir of enteric pathogens” (Santamaria & Toranzos 2003).

The 2007 Census of Agriculture data for American Indian Reservations listed a total of 389 farms with livestock on the Crow Reservation and a total inventory of about 105,000 cattle and calves (USDA 2009). Cattle grazing has historically impacted water quality throughout the western USA (Derlet et al. 2010). Cattle manure directly deposited alongside riverbanks is easily washed into rivers by rainfall and during the spring snowmelt season. Given our observations of ranching operations within the Little Bighorn watershed, and the measurement of significant levels of *E. coli* in the Little Bighorn River during spring runoff and early summer, we hypothesized that the Little Bighorn River was contaminated with *E. coli* O157:H7 and related EHEC serotypes.

Samples from the Little Bighorn River were screened for the presence of O157:H7 and related nonO157:H7 EHEC serotypes by testing *E. coli* isolates for the intimin and Shiga toxin virulence genes during the spring runoff seasons of 2008 and 2009. After successful isolation of EHEC, the Crow Environmental Health Steering Committee requested that a section of the Little Bighorn River close to the Wyoming border and running through pastureland associated with a 9000 head concentrated animal feeding operation (CAFO) be studied. CAFOs in general are known to generate very large amounts of manure that frequently serve as reservoirs for bacterial pathogens including *E. coli* O157:H7 (Smith & Perdek 2003; Gerba & Smith 2005; USEPA 2005b). A fecal microbial source-tracking study was initiated in 2010 to compare isolates of *E. coli* obtained from the Little Bighorn River with isolates from CAFO manure samples, to test the hypothesis that river water isolates of *E. coli* are linked to cattle in the CAFO ranch area.

During the microbial source tracking phase of our study, cattle-associated serotypes for two of the pathotypes of *E. coli* involved in waterborne, diarrheal disease were investigated. The EHEC group exemplified by serotype O157:H7 is evolving rapidly to give rise to variant strains causing outbreaks of increasingly severe disease (Manning et al. 2008). While enteropathogenic *E. coli* (EPEC) are not generally as important in causing diarrheal disease in more developed countries, there is a subgroup of EPEC termed atypical EPEC which: (1) appears to more closely resemble EHEC than typical EPEC in terms of genetics and virulence properties; (2) like EHEC, can use animals such as cattle as a reservoir; and (3) like EHEC, appear to be emerging pathogens in developed countries (Trabulsi et al. 2002). EHEC, EPEC, and atypical EPEC share a

major virulence determinant, the locus of enterocyte effacement (LEE) pathogenicity island (PI), comprised of the intimin gene and auxiliary genes which facilitate intimate attachment to host intestinal epithelial tissue (Donnenberg & Whittam 2001; Schmidt 2010; Bolton 2011). Presence of the LEE PI in these pathotypes provided the rationale for screening isolates matched by fingerprinting for the *eae* gene, and in turn identifying serotypes of these important *E. coli* pathotypes present in our study area. Screening for *eae* and serotyping also provided a test of the validity of matching *E. coli* isolates by DNA fingerprinting.

## Materials and methods

### *Virulence gene testing, 2008–2009*

Little Bighorn River water samples were collected in sterile containers in the town of Crow Agency on 25 May, 1 June, and 30 June 2008, for a total of 10 individual samples. During 2009, samples were collected at two locations, once a week, over a five-week period from 27 May to 26 June, for a total of 10 individual samples. These two locations were referred to as swim hole, located on the east bank of the river at a popular swimming spot in the Crow Fair campgrounds (45°36'1" N, 107°26'12" W), and storm drain, located on the west bank of the river 0.25 mile downstream of the swim hole and immediately downstream of the bridge near LBHC (45°36'12" N, 107°27'14" W). Water samples were stored in a cooler with icepacks and transported back to MSU for processing within 24 h.

Measured volumes of water samples were filtered through membrane filters (Millipore HAWG047S6). The manufacturer's protocol for using the m-ColiBlue24 methods states that samples ideally produce between 20 and 80 colonies total per membrane. Volumes of between 5 and 100 mL were filtered, depending on factors that could influence bacterial concentration in the river (e.g. recent rain and snowmelt events that would wash manure or soil-associated bacteria into the river, proximity to livestock). All samples were filtered and plated in triplicate. Filters were placed in 50 mm culture plates on top of filter pads pre-saturated with 2 mL of m-ColiBlue24 medium (Hach) following manufacturer's instructions and grown overnight at 37 °C to allow enumeration of coliform and *E. coli* bacteria. Concentrations were expressed as colony forming units (CFU) per 100 ml of water.

After enumeration, presumptive *E. coli* colonies (blue in color) were restreaked onto CHROMagar O157 (CHROMagar, Paris, France) plates. The CHROMagar O157 plates were incubated overnight at 37 °C and any mauve-colored colonies were restreaked for isolation onto CHROMagar O157 plates. Positive (mauve) colonies were restreaked a third time to isolate pure strains. Individual isolates were assigned unique identifying codes. Isolates were regrown on R2A agar (Difco) plates overnight at 37 °C. Colonies were picked and resuspended in 200 ul sterile water. DNA was released by boiling for 10 minutes and tested using polymerase chain reaction (PCR) protocols for three virulence genes (intimin – *eae*, Shiga toxin 1 – *stx1*, and Shiga toxin 2 – *stx2*) characteristic of EHEC. The PCR primers used were as described by (Chakraborty et al. 2001):

*eae* forward primer: 5'-AAACAGGTGAAACTGTTGCC-3'

*eae* reverse primer: 5'-CTCTGCAGATTAACCCTCTGC-3'

*stx1* forward primer: 5'-CAACACTGGATGATCTCAG-3'

*stx1* reverse primer: 5'-CCCCCTCAACTGCTAATA-3'

*stx2* forward primer: 5'-ATCAGTCGTCACTCACTGGT-3'

*stx2* reverse primer: 5'-CTGCTGTACAGTGACAAA-3'

(Note: All PCR primers were custom synthesized by Integrated DNA Technologies). PCR was performed using a LA Taq polymerase kit (Takara) and 10 µl of the DNA-containing supernates (final reaction volumes of 25 µl) for 35 cycles using the following parameters: 60 s @ 94 °C (denaturation), 30 s @ 57 °C (annealing), and 30 s @ 72 °C (extension).

PCR products were analyzed by electrophoresis using 1.2% agarose gels. The expected amplicon sizes were 454 bp for the *eae* gene, 350 bp for *stx1*, and 110 bp for *stx2* (Chakraborty et al. 2001). Positive controls were run using a strain of *E. coli* O157:H7 (identification number DEC 3A, original strain number 3299-85; STEC Center 2012). Negative controls were run using a nonEHEC strain of *E. coli* that was an environmental isolate previously characterized by our laboratory group.

Strains that were positive for any of the three virulence genes were regrown on R2A agar overnight at 37 °C, and processed for a second round of PCR analysis and oxidase testing. API 20E (BioMérieux) strip identification was then performed to confirm that the PCR-positive, oxidase-negative isolates were *E. coli*. PCR-positive *E. coli* strains were sent to the Pennsylvania State University *E. coli* Reference Laboratory for serotyping.

### ***Watershed survey of the Upper Little Bighorn River, 2010***

A watershed survey of the Little Bighorn River, starting upstream from the bridge closest to the Wyoming-Montana state line (1st state line bridge) and ending downstream at the town of Wyola (see Table 1 for site locations), was conducted during the summer and autumn of 2010. All river sampling sites are described in Table 1. Immediately downstream of the 1st state line bridge is a US Geological Survey streamflow monitoring station (site designation USGS 06289000) providing data to a National Water Information System web interface (USGS 2012). Sampling sites were upstream, adjacent to, and downstream of the CAFO ranch-associated pasturelands. The town downstream of and closest to the CAFO ranch area, Wyola, was also included. Pass Creek, a small tributary stream just upstream of Wyola, was also sampled.

Sampling site selection was guided by the decision criteria for conducting a sanitary survey as presented in the USEPA's Microbial Source Tracking Guide Document (USEPA 2005a). Readily accessible sampling sites of interest were selected by automobile travel. Additional sites south of the CAFO feedlots were chosen after wading the river downstream from the CAFO ranch headquarters bridge and alongside the brushy, forested area where water from the CAFO drainage ditch enters the north bank of the river (Figure 1). Three possible drainage sites noted in Table 1 and Figure 1 were selected based on their potential to discharge water during rain and snowmelt conditions. These three sites were chosen since they were likely to reflect water quality most directly affected by CAFO feedlot drainage as opposed to CAFO ranch grazing activity and associated pastureland runoff. The flow of genetic markers (DNA fingerprints and virulence gene markers) that would test for transport of cattle feedlot-derived *E. coli* bacteria to the river was assessed by sampling these locations.

Table 1. Description of Little Bighorn River sampling sites (2011–2012). Sampling site abbreviations are used as part of the isolate ID naming.

Abbreviation for sampling location	Description of sampling site on or near the Little Bighorn River; including latitude/longitude	Driving distance in miles from the 1st state line bridge (most upstream site)
1st SLbr	1st state line bridge; 1st bridge down-stream of the Wyoming/Montana (WY/MT) state line (45°0'23" N, 107°36'56" W)	0
2nd SLbr	2nd state line bridge; 2nd bridge downstream of the WY/MT state line (45°2'59" N, 107°34'7" W)	4
SLRbr	CAFO ranch headquarters bridge; 3rd bridge downstream of the WY/MT border (45°3'17" N, 107°31'57" W)	6
lotE	CAFO feedlot E: manure sampled here	Feedlots are about 0.8 mile north of the CAFO drainage ditch – culvert sampling site Manure drainage is south of and directly fed from lot E
lotF	CAFO feedlot F: manure sampled here	
MD	Drainage canal receiving manure slurry from feedlot E during rain/snowmelt	
Cdd(culvert)	CAFO drainage ditch runs south from feedlots, leading to foliage/river bank; ditch is sampled at culvert under road (45°3'39" N, 107°31'30" W)	6.5 (drainage ditch sampling site is a culvert running under a dirt road ~200 yards north of the Little Bighorn River
1st Cdr	1st possible CAFO drainage site into Little Bighorn River, north bank (45°3'32" N, 107°31'39" W)	The 3 sites on the north bank of the river are all within ~200 yards south of the CAFO drainage ditch culvert. The ditch feeds into an area of thick foliage and small trees, which in turn may feed into the river during high rainfall or snowmelt events
2nd Cdr	2nd possible CAFO drainage site into Little Bighorn River, north bank (45°3'33" N, 107°31'31" W)	
3rd Cdr	3rd possible CAFO drainage site into Little Bighorn River, north bank (45°3'37" N, 107°31'20" W)	
SCdr	Irrigation ditch drainage site into Little Bighorn River on south bank (45°3'24" N, 107°31'20" W)	12
Black Pass	Black Bridge (45°6'3" N, 107°26'23" W) Pass Creek is a tributary stream of the Little Bighorn River (45°7'23" N, 107°24'16" W)	Pass Creek Bridge sampling site is about 0.25 mile south of the creek/river confluence
Wyola Swim	Wyola (45°7'52" N, 107°24'9" W) Crow Fair swim hole (45°36'1" N, 107°27'12" W)	15 Crow Agency is 37 miles north of Wyola by road

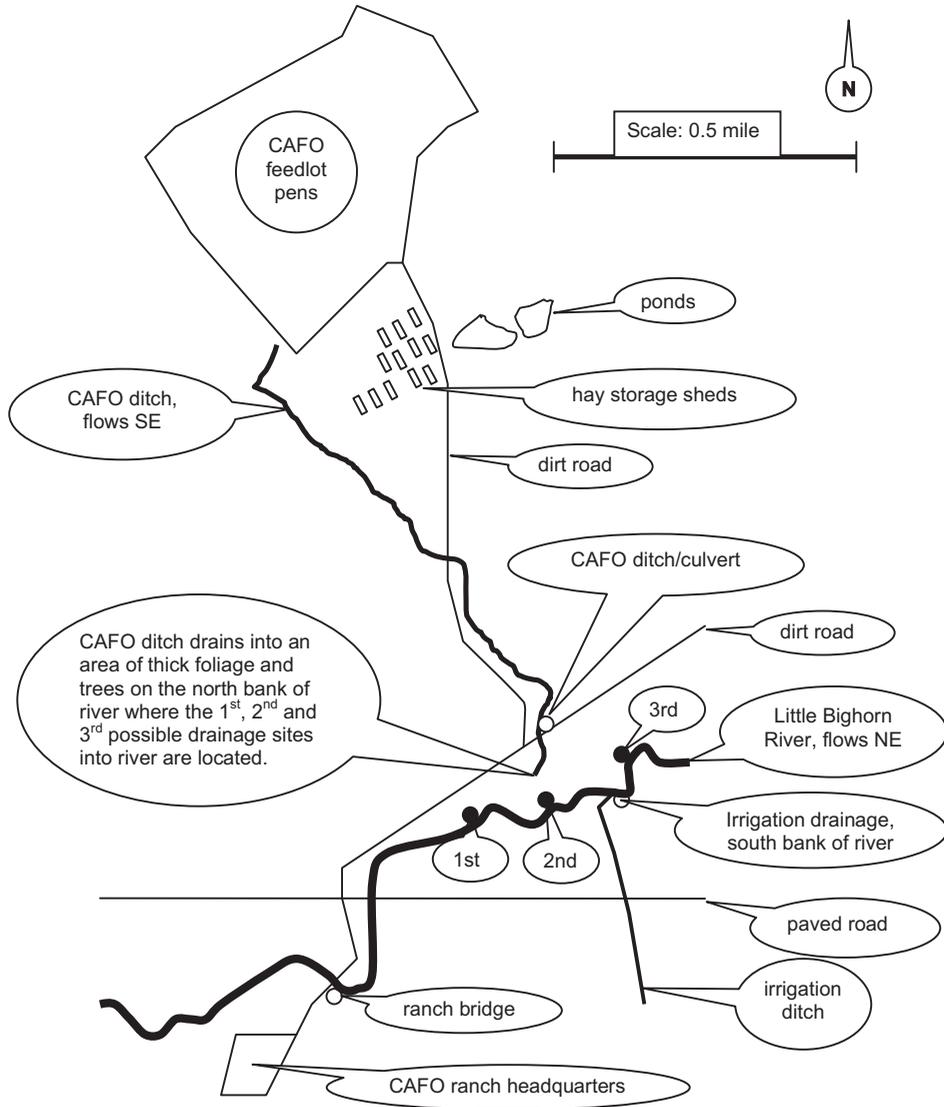


Figure 1. Map of the CAFO study area. The Little Bighorn River flows from the southwest to the northeast.

### ***Microbial source tracking study protocols, 2011–2012***

River sampling for source tracking was conducted before spring runoff and through summer seasons of 2011 and 2012. Water and manure samples were collected in sterile containers at the sampling locations described in Table 1. Containers were placed on ice packs for transportation back to MSU. A small amount of manure was transferred to a sterile tube containing 10 ml of sterile water, dispersed by vortexing, and the dispersed mixture allowed to settle for 30 min to remove large particles. Water with as little visible manure debris as possible was then transferred to a clean tube and processed for growth on m-ColiBlue24 as described above. River water and manure-derived samples

were initially processed as described above for virulence gene testing, except that *E. coli* colonies grown on m-ColiBlue24 were restreaked onto R2A agar and grown overnight at 44 °C. The elevated temperature was chosen to select for thermotolerant *E. coli* found in environmental sources that are more likely to have been derived from mammalian hosts (see Feng et al. 2002).

After restreaking, colonies were picked and resuspended in 200 µl sterile water and DNA prepared by the boiling method as before. Bacterial isolates were assigned individual codes or names using a three-part convention of sampling date, sampling site abbreviation (see Table 1), and sequential numbering. DNA concentrations were measured using a Nanodrop spectrometer and diluted to a standard concentration of 5 ng/µl. All DNA preparations were tested by PCR for the β-glucuronidase (*uid*) gene for presumptive identification of *E. coli*. Primers used were as described by Ram et al. (2004):

*uid* forward: 5'-AATAATCAGGAAGTGATGGAGCA-3'

*uid* reverse: 5'-CGACCAAAGCCAGTAAAGTAGAA-3'

PCR was performed using the LA Taq polymerase kit and 10 µl of the DNA-containing supernates (final reaction volume of 25 µl) for 35 cycles using the following cycling parameters: 60 s @ 94 °C, 30 s @ 60 °C, and 30 s @ 72 °C. PCR products were analyzed by agarose gel electrophoresis to identify isolates generating a 587-nucleotide β-glucuronidase amplicon.

DNA fingerprinting of *E. coli* isolates was performed using enterobacterial repetitive intragenic consensus (ERIC) sequence primers (Versalovic et al. 1991):

ERIC-1R: 5'-ATGTAAGCTCCTGGGGATTAC-3'

ERIC-2: 5'-AAGTAAGTGAAGTGGGGTGAGCG-3'

Each 25 µl reaction mixture contained 10 µl (50 ng) of isolate DNA and 15 µl of a master mix of LA Taq reagents and primers. Per reaction, the master mix contained 0.25 µl Taq enzyme, 2.5 µl buffer, 4 µl dNTP mix, 1 µl 0.3 µg/µl ERIC1R, 1 µl 0.3 µg/µl ERIC2, and 6.25 µl water. All PCR for DNA fingerprinting was run using a single Eppendorf model 5331 thermocycler and the following parameters: 95 °C for 2 min, and 30 cycles of 94 °C for 3 s, 92 °C for 30 s, 52 °C for 1 min, and 65 °C for 8 min (modified from Rademaker & Bruijn 1997; McLellan et al. 2003).

For DNA fingerprinting, agarose gel electrophoresis was conducted using 1.2% gels in 0.5× TBE buffer (60 ml gel volume in a 9 cm × 11 cm tray, Owl Scientific) run at ~110 volts for 2 h 15 min. Gels were stained with ethidium bromide for 15 min for fingerprint image capture.

Gel images were processed and analyzed using GelComparII software (Applied Maths). Curve-based Pearson correlation coefficient (1% optimization) was used to calculate similarities between fingerprints. Multiple runs of an *E. coli* O157:H7 reference strain gave similarity calculations ranging between 92 and 98%, with a mean value of 95%. Accordingly, a cut-off value of 95% similarity was used to give a first approximation of fingerprint pairs that were considered likely to be identical.

All isolates were tested by PCR for the *eae* gene using the protocol described above. Serotyping of *eae*<sup>+</sup> isolates was performed at the Pennsylvania State University *E. coli* Reference Laboratory.

## Results

### *Isolation of eae+/stx+ E. coli during 2008–2009*

During the spring runoff season in May and June of 2008, a total of 10 water samples were collected from the Little Bighorn River in the town of Crow Agency. Thirteen bacterial isolates collected on 30 May from the river water grew as mauve colonies on CHROMagar O157 medium. One isolate from the Little Bighorn River at the Crow Fair swim hole site was positive for the intimin and Shiga toxin 1 genes. Testing by the Pennsylvania State University *E. coli* Reference Laboratory identified this isolate as serotype O111:H8 (Table 2).

Spring runoff sampling of the river was continued during a five-week period, from May 27 through 26 June 2009. A total of 10 water samples were collected in Crow Agency at two sites on the Little Bighorn River, at the Crow Fair swim hole and 0.25 mile downstream at the storm drain located north of LBHC. Sixty-nine isolates grew as mauve colonies on CHROMagar O157. In testing for the virulence genes *eae*, *stx1*, and *stx2* indicative of EHEC, nine of these *E. coli* isolates tested positive for *eae* and at least one of the two *stx* genes. One of these isolates was of serotype O157:H7; all serotyping results for these isolates are presented in Table 2.

### *E. coli source tracking results, 2010–2012*

Some general observations of field conditions are relevant to interpreting the source-tracking study results. The upper Little Bighorn River watershed is home to deer and several other species of mammals and birds that can serve as nonpoint sources of fecal contamination of the river. Small groups of cattle were frequently observed on pastureland located between the 1st and 2nd state line bridge, sometimes grazing close to the river without setbacks. During the watershed survey conducted in 2010, livestock were grazing close to the river (with and without setbacks) along the seven-mile length of the Little Bighorn River between the CAFO ranch headquarters and Black Bridge. Several small family ranches are also found along this length of the river, and one family ranching operation had indications of domestic geese and sheep having ready access to the river. In summary, a variety of domestic livestock from both CAFO and nonCAFO lands could serve as nonpoint sources of fecal contamination of the river downstream from the CAFO feedlot area.

Source tracking sampling was commenced in 2011 and focused on the pre-spring runoff through summer season. Sampling of the watershed on 11 May was conducted after a few days of steady rainfall, an event that coincided with increased stream flow and water level as indicated by USGS streamflow measurements (monitoring site 06289000 data from USGS 2012). There was a marked increase in *E. coli* levels measured for 11 May samples compared to the previous month's sampling (Table 3), suggesting that this rainfall event coincided with and marked the beginning of runoff season. The 3rd CAFO drainage site had water running from the bank directly into the river. The concentration of *E. coli* measured in the water collected directly from this bank drainage on 11 May (933 CFU/100 ml, Table 3) was much higher than the concentrations measured just upstream at the 1st and 2nd possible drainage sites (20–21 CFU/100 ml, Table 3), suggesting that accumulated bacteria were being flushed from the soil by high water. In late May of 2011, severe flooding affected residents of the Crow Indian reservation. The flooding temporarily prevented travel to the reservation, and limited access to and sampling of the Little Bighorn River in the region bordering

Table 2. Summary of *E. coli* strains isolated from the Little Bighorn River in the vicinity of the Crow Fair swim hole and college bridge storm drain, Crow Agency, during 2008, 2009, and 2012. Serotyping was performed by the Pennsylvania State University *E. coli* Reference Laboratory.

Serotype (date & location of sampling)	<i>eae</i>	<i>stx1</i>	<i>stx2</i>	Association with human disease?
O11:H8 (30 May 2008; swim hole)	+	+	–	EHEC, bloody diarrhea & HUS (Brooks et al. 2004)
O157:H7 (3 June 2009; storm drain)	+	+	+	Prototypic EHEC of major public health concern
O2:H27 (18 June 2009; storm drain)	+	+	+	Reported in human disease (Monahan et al. 2011)
O?:H38 (18 June 2009; storm drain)	+	+	+	Unknown
O18:H? (18 June 2009; swim hole)	+	+	+	?
O18:H49 (18 June 2009; swim hole)	+	+	+	?
O89:H+ 2 isolates (18 June 2009; swim hole)	+	+	+	?
O4:H16 (18 June 2009; swim hole)	+	+	+	?
O154:H7 (18 June 2009; swim hole)	+	+	+	?
O5:H– (18 May 2012; swim hole)	+	+	–	EHEC, bloody diarrhea (McLean et al. 2005)

Note: It is not known whether any of these environmental isolates are capable of causing disease.

Table 3. 2011 Little Bighorn River *E. coli* counts (CFU/100 ml).

Site	21 March	29 April	11 May (before flood)	27 May (one week after flooding)	21 June	July 8	22 July	10 August	30 August
River, 1st stateline bridge	0	0	0.3	5	4	1	No testing	3	7
River, 2nd stateline bridge	0.3	0	15	12	7	8	No testing	29	7
River, Sunlight Ranch Bridge	No testing	3	97	12	11	18	No testing	24	21
CAFO drainage ditch culvert, roadside	0.3	47	1510	203	210	423	No testing	70	27
River, 1st possible CAFO drainage, north	0.3	4	20	Not tested, water too high	Not tested, high water	Not tested, high water	No testing	No testing	7
River, 2nd possible CAFO drainage, north	0.3	0	21	Not tested, water too high	Not tested, high water	Not tested, high water	No testing	No testing	7
River, 3rd possible CAFO drainage, north	2	0	993 (water from bank)	Not tested, water too high	Not tested, high water	Not tested, high water	No testing	No testing	6
River, irrigation ditch drainage, south bank	11	17	134	Not tested, water too high	Not tested, high water	Not tested, high water	No testing	No testing	No testing
River, Black Bridge	0	0	135	32	26	34	No testing	28	11
Pass Creek Bridge	No testing	60	400	340	30	150	No testing	99	32
River, Wyola	14	13	493	193	38	65	No testing	55	17
River, Crow Fair swim hole	13	30	683	560	75	321	93	426	47

the CAFO drainage. During our sampling trips in June and July, high water levels made it too dangerous to wade and collect water samples at the river sampling sites (1st, 2nd, and 3rd possible CAFO drainage sites on the north bank of the river – see map of Figure 1) closest to the CAFO feedlots.

Manure was collected from the CAFO feedlots (corrals E and F) on 29 April and 11 May 2011, for *E. coli* isolation and fingerprinting. By the next sampling date of 21 June, the feedlots were empty of cattle.

During the summer months, children frequently use the Crow Fair swim hole for recreation. Swim hole samples collected on 8 July and 10 August 2011 had *E. coli* concentrations of 321 and 426 CFU/100 ml, respectively, both exceeding the EPA guideline for recreational use (Table 3).

Three sampling trips were made to the Wyola-CAFO study area during the runoff season of 2012 (13 April, 18 May, and 27 June). A review of USGS streamflow data (monitoring site 06289000 data, from USGS 2012) indicated that the first two trips were made as runoff stream flows and levels were increasing, and the third after runoff had peaked. During the May 18 trip, water levels were too dangerous for wading along the river's edge to sample at the 1st, 2nd, and 3rd possible CAFO drainage sites. Because of the flooded bank event noted on May 11 of the previous year, the 3rd site was instead approached by hiking/wading directly from the roadside CAFO drainage ditch culvert through very dense foliage and forest. Much of this resembled a flooded wetland. Advantage was taken of the flooded conditions by sampling from a logjam area of pooled but flowing water, and also from a stream of water spilling from the bank at the 3rd possible CAFO drainage site.

During 2011 sampling of the Wyola-CAFO study area and the distant Crow Fair swim hole in Crow Agency, measurements of *E. coli* were highest on 11 May (Table 3). During the more limited sampling conducted in 2012, *E. coli* measurements were also highest in May during early spring runoff (Table 4).

A total of 167 DNA fingerprints were generated by ERIC PCR of manure lot and manure drainage canal *E. coli* isolates obtained from sampling in April and May, 2011. These fingerprints constitute the manure “source library” in a classic library-dependent source tracking study. Table 5 presents a summary of sampling dates and locations for all manure and water isolates that were fingerprinted. During both 2011 and 2012, and specifically in the Wyola-CAFO study area, a total of 196 fingerprints were generated from *E. coli* isolated from Little Bighorn River sites, 84 fingerprints from CAFO drainage

Table 4. 2012 Little Bighorn River *E. coli* counts (CFU/100 ml).

Site	13 April	18 May	27 June
River, 1st stateline bridge	0.3	8	1
River, 2nd stateline bridge	7	53	15
River, CAFO ranch headquarters bridge	8	96	13
CAFO drainage ditch culvert (not part of river)	0	93	129
Wetland/logjam area above 3rd possible CAFO drainage, feeding river	No testing	756	25
Wetland/3rd possible CAFO drainage, feeding river	No testing	93	18
River, Black Bridge	0	246	35
Pass Creek Bridge	35	668	97
River, Wyola	10	305	64
River, Crow Fair swim hole	3	172	103

Table 5. Summary of *E. coli* isolate fingerprints analyzed.

Isolate series (sampling date and site abbreviation)	Number in group	Sampling site/source description
2011-4-29 lotE	31	Manure from CAFO feedlot E
2011-4-29 lotF	42	Manure from CAFO feedlot F
2011-5-11 1st SLbr	1	River
2011-5-11 2nd SLbr	5	River
2011-5-11 SLRbr	1	River
2011-5-11 lotE	32	Manure from CAFO feedlot E
2011-5-11 lotF	32	Manure from CAFO feedlot F
2011-5-11 MD	30	Manure drainage canal from CAFO feedlot E
2011-5-11 Cdd	7	Drainage ditch culvert
2011-5-11 1st Cdr	11	River
2011-5-11 2nd Cdr	12	River
2011-5-11 3rd Cdr	12	Flooded, water streaming over bank into river
2011-5-11 SCdr	12	River
2011-5-11 Black	12	River
2011-5-11 Pass	4	Tributary to Little Bighorn River
2011-5-11 Wyola	8	River
2011-5-11 swim	3	River/swim hole, Crow Agency
2011-6-21 Cdd	17	Drainage ditch culvert
2011-6-21 Black	19	River
2011-7-8 Cdd	20	Drainage ditch culvert
2011-7-8 Black	29	River
2012-4-13 1st SLbr	1	River
2012-4-13 2nd SLbr	14	River
2012-4-13 SLRbr	23	River
2012-4-13 Pass	20	Tributary to Little Bighorn River
2012-4-13 Wyola	9	River
2012-4-13 Swim	1	River
2012-5-18 Cdd	40	Drainage ditch, roadside culvert
2012-5-18 3rd Cdr	40	Flooded, water streaming over bank into river
2012-5-18 logjam	40	Flooded wetland area, ~ 50 yards north of 3rd Cdr
2012-5-18 Black	17	River
2012-05-18 Pass	22	Tributary to Little Bighorn River
2012-05-18 Wyola	22	River
	589 total	

ditch culvert isolates, 46 from Pass Creek isolates, 52 from isolates from the water streaming off the bank at the 3rd possible CAFO drainage, and 40 from isolates from the logjam/flood area (close to the 3rd possible CAFO drainage site). Four fingerprints were also obtained for Crow Fair swim hole isolates.

Of the 589 fingerprints analyzed, a total of 170 DNA fingerprint pairs were identified as having similarity values of 95% or greater. The majority of these pairs were among isolates sampled from the same site on the same day. Twenty-four of the fingerprint pairs were matches between a manure isolate (i.e., a member of the source library) and a river water isolate, a manure isolate and a CAFO drainage ditch water isolate, or a CAFO drainage ditch isolate and a river isolate (Table 6).

Isolates that tested positive for the intimin gene were sent to the Pennsylvania State University *E. coli* Reference Laboratory for additional serotype testing. Among the 38 manure isolates tested, two dominant serotypes were evident: 19 isolates were identified as O156:H8, and eight were identified as O15:H2 (see Table 7 for a complete summary of all serotype assignments). Serotyping for two *ea*<sup>+</sup> water isolates from the May 18,

Table 6. Fingerprint pairs having similarity values of 95% or higher that suggest drainage migration of bacteria from CAFO feedlot manure into the Little Bighorn River. Matched pairs whose river isolate was collected at a site in the immediate vicinity of possible CAFO drainage into the river are highlighted in italics. The two pairs that include a manure isolate-CAFO drainage ditch (water) isolate match (pair #30) and a CAFO drainage isolate-river isolate match (pair #153) are also highlighted. DNA fingerprints were generated by ERIC PCR; similarity values were calculated using curve-based Pearson correlation coefficient. Fingerprint naming assignments are made up of sampling date, sampling site abbreviation, followed by sequential numbering.

Pair #	1st fingerprint of pair	2nd fingerprint of pair	Similarity values (%)
27	2011-4-29 lotF 12	2012-5-18 Wyola 8	96
28	2011-4-29 lotF 12	2012-5-18 Wyola 16	95
29	2011-4-29 lotF 35	2011-5-11 Wyola 6	95
30	<i>2011-4-29 lotF 41</i>	<i>2011-7-8 Cdd 49</i>	95
31	2011-4-29 lotF 45	2011-6-21 Black 18	95
32	2011-4-29 lotF 45	2011-7-8 Black 13	95
33	<i>2011-4-29 lotF 45</i>	<i>2011-7-8 Black 17</i>	95
40	<i>2011-5-11 lotE 19</i>	<i>2011-5-11 2ndCdr 10</i>	95
41	2011-5-11 lotE 19	2011-5-11 Black 10	96
42	2011-5-11 lotE 19	2011-5-11 Wyola 6	96
43	<i>2011-5-11 lotE 20</i>	<i>2011-5-11 SCdr 6</i>	96
44	<i>2011-5-11 lotE 21</i>	<i>2011-5-11 2ndCdr 10</i>	95
45	2011-5-11 lotE 21	2011-5-11 Black 10	95
46	2011-5-11 lotE 21	2011-5-11 Wyola 6	96
58	<i>2011-5-11 MD 22</i>	<i>2011-5-11 SCdr 11</i>	98
59	2011-5-11 MD 22	2011-5-11 Wyola 8	96
60	2011-5-11 MD 25	2011-5-11 Black 10	95
61	<i>2011-5-11 MD 31</i>	<i>2011-5-11 SCdr 10</i>	96
81	2011-6-21 Cdd 11	2011-6-21 Black 16	96
88	2011-7-8 Cdd 9	2011-7-8 Black 13	95
89	2011-7-8 Cdd 11	2011-7-8 Black 8	95
90	2011-7-8 Cdd 11	2011-7-8 Black 17	95
91	2011-7-8 Cdd 13	2011-7-8 Black 11	95
153	<i>2012-5-18 Cdd 35</i>	<i>2011-5-11 2ndCdr 6</i>	95

2012 sampling was also performed. One of these, an isolate obtained from water running from the flooded bank into the river at the third possible CAFO drainage, was confirmed to be O156:H8, the same as the most prevalent manure isolate serotype (Table 7). The second May 18 water isolate, obtained from the distant Crow Fair swim hole, was identified as serotype O5:H- (see Table 1).

Assignment of serotypes allowed for an additional test of whether fingerprints assigned as matching pairs on the basis of high similarity values were indeed identical on the basis of serotyping. Where possible, members of matched pairs having a similarity equal to or greater than 95% were cross-referenced with their serotype assignments to check whether both members of the pair had the same serotype. Nine matching fingerprint pairs were identified as having one or both members of the pair assigned serotypes (Table 8). Of these nine pairs, four had identical serotypes and also shared an *eae+* profile (pairs 1, 35, 48, and 57, listed in Table 8). Two pairs (pairs 51 and 52, Table 8) had only one-member-assigned serotype but had differing *eae* genotypes; these pairs could be considered invalid matches. The three remaining pairs (pairs 17, 39, and 60, Table 8) having only one-member-assigned serotype had identical *eae+* genotypes. Subsequent testing of the previously nonserotyped members of these three pairs revealed unique serotypes, indicating that these pairs were also invalid matches. In summary, four of the

Table 7. Summary of *eae+* *E. coli* strains isolated from CAFO manure and manure drainage canal (during 2011) and from 3rd possible CAFO drainage into the Little Bighorn River (2012).

Isolation site (and date)	Serotype	Number	Association with human disease?
3rd possible CAFO drainage; water flowing from flooded bank into river, 1 mile from CAFO pens (May 18, 2012)	O156:H8	1	EPEC: role in disease not known; serotype has been reported in infants (Bokete et al. 1997)
Manure/drainage from CAFO pens (April/May 2011)	O156:H8	19	EPEC: diarrhea, HUS (Beutin et al. 2005) Unknown, but found in humans-asymptomatic bacteriuria (Roos et al. 2006) Diarrhea, hemorrhagic colitis (Tzipori et al. 1988) Unknown Reported in humans (Boerlin et al. 1999) Unknown Unknown Unknown Unknown Unknown
	O15:H2	8	
	O25:H2	3	
	O4:H-	1	
	O7:H8	1	
	O8:H+	1	
	O83:H7	1	
	O147:H21	1	
	O154:H7	1	
	O156:H14	1	
O-:H39	1		

Note: It is not known whether any of these isolates are capable of causing disease.

nine pairs, or 44%, of the pairs initially assigned as identical matches on the basis of high similarity were confirmed to be identical of the basis of serotyping and *eae* testing.

## Discussion

Concerns about water quality and high levels of *E. coli* found in the Little Bighorn River, especially during spring runoff season, as well as the presence of cattle and other livestock frequently found to be grazing without setbacks on the banks of the river, prompted screening for EHEC and related bacteria beginning in the spring of 2008. During testing of *E. coli* isolated from the Little Bighorn River in the town of Crow Agency during May and June of 2008, an isolate of EHEC serotype O111:H8 was identified. The O111:H8 serotype has been associated with human disease, having caused a well-publicized outbreak of bloody diarrhea and HUS among participants at a high school cheerleading camp in Texas in 1999 (Brooks et al. 2004). A related O111:H- (nonmotile) strain was also implicated in an outbreak of diarrhea and (HUS) affecting over 300 people in Oklahoma in 2008 (Bradley et al. 2012). In response to a growing number of disease outbreaks involving nonO157:H7 serotypes of Shiga toxin-producing *E. coli*, the O111 serotype has been included in a 2011 listing of nonO157, Shiga toxin-producing serogroups, including O26, O45, O103, O121, and O145; since 2011, the USDA Food Safety and Inspection Service has required inspection of beef supplies for these pathogens (USDA 2011).

During 2009 testing, nine additional strains of *E. coli* harboring intimin and Shiga toxin genes were isolated from the Crow Fair swim hole and nearby storm drain. Isolates included one strain of O157:H7 as well as one strain of O2:H47, a serotype described as

Table 8. Validation test of select fingerprint pair matches from Table 6 by cross-reference with serotype assignments and *eae* profile. Matching pairs that had one or both isolates positive for *eae* were sent to the Pennsylvania State University *E. coli* Reference Lab for serotyping.

Pair #	1st fingerprint of pair	2nd fingerprint of pair	Similarity values, validation check
1	2011-4-29 lotE 2 serotype O156:H8, <i>eae</i> +	2011-4-29 lotE 8 serotype O156:H8, <i>eae</i> +	95%, same serotype, both <i>eae</i> +
17	2011-4-29 lotF 19 serotype O156:H8, <i>eae</i> +	2011-4-29 lotF 20 serotype O83:H7, <i>eae</i> +	96%, but different serotype
35	2011-5-11 lotE 3 serotype O156:H8, <i>eae</i> +	2011-5-11 lotE 4 serotype O156:H8, <i>eae</i> +	99%, same serotype, both <i>eae</i> +
39	2011-5-11 lotE 19 serotype O160:H30, <i>eae</i> +	2011-5-11 MD 25 serotype O154:H7, <i>eae</i> +	98%, but different serotype
48	2011-5-11 lotF 22 serotype O15:H2, <i>eae</i> +	2011-5-11 lotF 23 serotype O15:H2, <i>eae</i> +	96%, same serotype, both <i>eae</i> +
51	2011-5-11 MD 4 serotype O156:H8, <i>eae</i> +	2011-5-11 MD 5 <i>eae</i> -	97%, but different <i>eae</i> genotype
52	2011-5-11 MD 4 serotype O156:H8, <i>eae</i> +	2011-5-11 MD 9 <i>eae</i> -	95%, but different <i>eae</i> genotype
57	2011-5-11 MD 26 serotype O156:H8, <i>eae</i> +	2011-5-11 MD 30 serotype O156:H8, <i>eae</i> +	97%, same serotype, both <i>eae</i> +
60	2011-5-11 MD 25 serotype O154:H7, <i>eae</i> +	2011-5-11 Black 10 serotype O8:H16, <i>eae</i> +	95%, but different serotype

an emerging pathogen (Monahan et al. 2011). In testing of 40 *E. coli* isolates from the May 18, 2012 sampling of the Crow Fair swim hole, one strain of O5:H<sup>-</sup> was found. The O5:H<sup>-</sup> serotype has also been associated with severe diarrheal disease in humans (McLean et al. 2005). It is not known whether any of these environmental river isolates of *E. coli*, positive for intimin and Shiga toxin genes, are capable of causing disease in humans. Presence of high levels of *E. coli* measured in the Little Bighorn River during late spring and early summer are most likely due to seasonal, spring runoff events which include heavy rainfall and snowmelt. Manure deposited along riverbanks, and soil-associated bacteria derived from manure, are readily washed into the river by rain and melting snow, giving rise to high levels of potentially pathogenic *E. coli*, including the disease-related serotypes detected in this study. It is of great concern that these potentially pathogenic strains were isolated during spring runoff from an area of the Little Bighorn River that is used extensively by children for swimming later in the summer when high water levels have subsided.

A total of 589 DNA fingerprints were processed for *E. coli* isolates collected during the 2011–2012 source tracking sampling. Among these, 170 pairs showed similarity values of 95% or greater. Among these matched pairs, 24 of the matches are consistent with the migration of bacteria from the manure lots to the river (Table 6). Of these 24 pairs, there were 17 pairs that included a manure isolate (i.e. from the source library) matched with a Little Bighorn River isolate, one pair that matched a manure isolate with a CAFO drainage ditch isolate, and six pairs that matched a CAFO drainage ditch isolate with a river isolate. Six of the fingerprint pairs listed in Table 6 (highlighted in the table) include a river isolate collected in the proximity of the CAFO drainage area, and one pair matches a manure isolate with a CAFO drainage ditch isolate (also highlighted in the table). These data are in strong agreement with the hypothesis that some river isolates of *E. coli* originate by migration from CAFO feedlot manure. The situation is complicated, however, in that CAFO feedlot cattle are also grazed in pastureland adjacent to the river upstream and downstream of the CAFO feedlot area, as seasonal conditions permit. These cattle deposit manure in pastureland along the river in a nonpoint source manner. Additionally, it is unknown whether wild animals or other nonCAFO ranch cattle on smaller farms in the Wyola-CAFO watershed harbor *E. coli* populations sharing high fingerprint similarity with the CAFO manure isolates. Considering these factors, it is acknowledged that the river isolates listed but not highlighted in Table 6 collected at either Black Bridge or Wyola might have originated from manure deposited downstream of the CAFO feedlot area. Conversely, downstream manure deposits may contain bacteria sharing DNA fingerprints similar to those of CAFO manure fingerprints. More extensive sampling of pastureland manure samples from both CAFO-associated and nonCAFO pastures are needed to create additional DNA fingerprint source libraries to address these issues. As noted by others, library composition and size is an important factor in execution of a source tracking study (Johnson et al. 2004). Our sampling of CAFO feedlot manure was relatively limited and unlikely to have captured all of the genetic diversity present in feedlot manure.

Despite the limited number and sources of samples, there are a few noteworthy aspects of the fingerprinting data to consider. First, one matched pair of fingerprints is between a manure isolate and a CAFO drainage ditch isolate (see Table 6, pair #30). The CAFO drainage ditch culvert is located between the CAFO feedlot area and the Little Bighorn River, and is about 0.8 mile south and downstream of the feedlot area. This finding is consistent with the hypothesis that CAFO feedlot manure-derived bacteria can be transported from the feedlot to the forested area on the north bank of the river via the drainage ditch.

A second feature comes from closer examination of the fingerprint pairs matching a manure or drainage ditch isolate with a river isolate (Table 6). Seventeen of these are pairs which include a river isolate taken from Black Bridge or Wyola, making it possible that the river isolates could have come from smaller farms located downstream of the CAFO area. Five of the pairs, however, include as the river isolate bacteria taken from the 2nd possible CAFO drainage site (pairs 40 and 44 from Table 6) and the south possible CAFO drainage (pairs 43, 58, and 61 from Table 6). These sites would be unaffected by farms and ranching activity occurring downstream. These data are consistent with the hypothesis that the river isolates may have arisen from the CAFO feedlot manure, although it is possible that some could have been derived from manure droppings from wild animals/birds or from cattle grazing upstream.

Among the 167 manure isolates that were DNA fingerprinted, 38 that were *eae+* were subsequently serotyped. Two dominant serotypes were evident: 19 of the isolates were of serotype O156:H8 and 8 were of serotype O15:H2 (Table 7). Serotype O156:H8 has been associated with infantile bloody diarrhea, but a role for this serotype in etiology of the disease has yet to be elucidated (Bokete et al. 1997). O15:H2 is a serotype of EPEC that is known to cause diarrhea and HUS (Beutin et al. 2005). It is not known whether any of our bovine manure isolates can cause disease in humans.

Serotyping provided another contribution to the source-tracking data. An isolate of serotype O156:H8, the dominant serotype found among the manure strains isolated in 2011, was also isolated from the water flowing into the river at the 3rd possible CAFO drainage site on May 18, 2012. This finding is consistent with the flow of manure-derived bacteria from the CAFO feedlots, down the CAFO drainage ditch, and into the forested area, where bacteria could be washed into the river during high water events.

Serotype data also provided a means of confirming DNA fingerprinting in identifying isolates as being highly similar or clonal in origin. Several members of matched DNA fingerprint pairs were serotyped after being identified as *eae+* (see Table 8 for the cross-referencing of serotyped fingerprints with fingerprint pairs). Matching of four of nine pairs (or 44%) of DNA fingerprints was corroborated by the serotype data.

The finding that *eae+* strains comprised 23% of the manure isolates suggests that cattle waste may effectively serve as a large and abundant reservoir and vector for EPEC bacteria. Exposure to environmental sources of these bacteria may have more serious public health consequences than just diarrheal disease, in that recent research has been investigating the role of EPEC in etiology of colon cancer (Maddocks et al. 2009). In human disease, EHEC and EPEC bacteria expressing the intimin gene attach to colonic mucosal tissue and cause an attaching-and-effacing pathology (Nataro & Kaper 1998). Infection by EPEC bacteria is a major cause of life-threatening infantile diarrhea in developing countries (Levine & Edelman 1984). Although diarrheal disease caused by EPEC has declined in developed countries, studies in Europe and Australia indicate that a significant proportion of up to 10% of otherwise healthy children harbor EPEC bacteria asymptotically (Beutin et al. 2003; Pabst et al. 2003). Persistence of asymptomatic EPEC infection through adulthood may be linked to development of colon cancer, given that a high percentage of colon cancer biopsies reveal adherent EPEC bacteria, and that EPEC appear to send biochemical signals to colon tissue that promote tumorigenesis (Maddocks et al. 2009).

The notable incidence of *eae* in manure isolates suggests that manure is not only a reservoir of pathogens, but also of the virulence genes themselves (Durso et al. 2011). *E. coli* in the river may not match isolates from the manure, but can potentially acquire virulence genes through horizontal gene transfer from manure isolates present in the

water, sediments, and biofilms (Kristiansson et al. 2011; Madsen et al. 2012). The spread of bacterial pathogens and associated virulence genes from cattle waste into the environment is of public health concern from the standpoint of evolution and emergence of new strains of pathogenic EHEC and EPEC (Loukiadis et al. 2006).

In summary, the identification of matched, “identical” pairs of *E. coli* by DNA fingerprinting and serotyping of *E. coli* isolates obtained from CAFO manure and river sampling support the hypothesis that CAFO manure may be a source of *E. coli* detected in the Little Bighorn River upstream of Wyola. The large amount of manure concentrated in the feedlot area, and the proximity of the feedlot area to what we have termed the CAFO drainage ditch, is of concern. Manure seepage may enter a ditch that flows south from the feedlot area down through a roadside culvert before draining into the forested area on the north bank of the Little Bighorn River. Detection of the *eae* virulence gene in 23% of the manure isolates tested during 2011 is of public health concern, given the potential of *eae*<sup>+</sup> strains of *E. coli* to colonize the intestines of humans, livestock, and wildlife and cause disease (Jerse & Kaper 1991; Vlisidou et al. 2006).

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