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MOLECULAR CHARACTERIZATION OF *ESCHERICHIA COLI* O157:H7 HIDE CONTAMINATION ROUTES – FEEDLOT TO HARVEST FLOOR

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Introduction

Harvest decontamination interventions (usually applied in multiple-hurdle systems) are more efficacious if the proportion of incoming cattle with *E. coli* O157:H7 on their hides is reduced. Cattle can be a reservoir for *E. coli* O157:H7 (Chapman et al., 1993) and these organisms can be transferred from hide to meat during slaughtering/dressing (Sofos et al., 1999) such that hides of animals being harvested can be a significant source of contamination of resulting carcasses (Riddell and Kerkeala, 1993; Bell, 1997; Sofos et al., 1999; Byrne et al., 2000; McEvoy at al., 2000). According to Ransom et al. (2003), carriage of *E. coli* O157:H7 on pre-evisceration carcasses was 7.1% if pre-harvest fecal prevalence was less than 20%, versus 12.5% if pre-harvest fecal prevalence was greater than 20%. Cattle hides may become contaminated with *E. coli* O157:H7 from feedlot pen floors (Dewell et al., 2003), contact between animals after departure from the farm (Tutenel et al., 2003), floors of lairage pens, or the stunning-box floor (Avery et al., 2002; Small et al., 2002, 2003).

Several studies have examined causality of carcass contamination at steps in the harvest process (Grau, 1987; Gill et al., 1996a, b; Bell, 1997; Gill and McGinnis, 1999), but such studies do not directly link sources with the introduction of specific organisms in terms of contamination events (Barkocy-Gallagher et al., 2001). The overall objective of this study was to identify genetic relationships of *E. coli* O157:H7 contamination found on cattle hides at the time of harvest, to *E. coli* O157:H7 found at associated pre-harvest sites, such as the feedlot, loading chutes, transport trailers, packing-plant holding pens, and feces obtained at various locations.

Materials and Methods

Samples were collected from Midwestern region commercial feedlots (N = 3), transporting cattle to packing facilities (N = 2), and Southwestern region feedlots (N = 3), transporting cattle to a Southwestern region packing facility. For each lot of cattle (defined as 1 feedlot shipment; N = 8), sets of ten sample types were collected: (1) feedlot pen floor composite, (2) feedlot pen water composite, (3) feed bunk and feed composites, (4) loading chute composite, (5) transport truck trailer wall and floor composite, (6) holding pen composite at beef plant, (7) plant pen water composite, (8) restrainer samples, (9) hide samples, and (10) corresponding colons. Sample sets were collected until at least 25 *E. coli* O157:H7 positive hides were confirmed as positive

(using biochemical means) for each of the Midwestern (N = 25) and Southwestern regions (N = 65).

Microbiological Analysis

Detection of *E. coli* O157:H7 in all samples was conducted according to the procedure in Barkocy-Gallagher et al. (2002).

Isolate confirmation was as follows; screening with the latex agglutination assay of the Oxoid *E. coli* O157:H7 Test Kit (Oxoid; Ogdensburg, NY), initial biochemical analysis of presumptive isolates (cellubiose, triple sugar iron (TSI) slants, and motility), screening with and O and H antigen agglutination test (RIM *E. coli* O157:H7 Latex test, Remel, Lenexa, KS), and the primary *E. coli* O157:H7 isolate from each sample also was subjected to VITEK analysis. Once confirmation testing was complete, isolates were stored in a 20% glycerol-TSB solution and frozen, pending molecular characterization testing.

Molecular Characterization of Recovered E. coli O157:H7 Isolates

Multiplex Polymerase Chain Reaction (PCR) of known *Escherichia coli* O157 gene targets (Gannon et al., 1997; Wang et al., 2002) and Pulsed-Field Gel Electrophoresis (PFGE) of restricted genomic DNA were used to identify recovered isolates.

Multiplex PCR

Each isolate was subjected to each of three multiplex PCR procedures, using different primers for amplification as follows: 1) for Shiga toxin genes 1 and 2 (stx_1 and stx_2) per Wang et al. (2002); 2) For enterohemorrhagic *E. coli* (EHEC) enterohemolysin (*hlyA*), *E. coli* somatic antigen O157 ($rfbE_{O157}$), and *E. coli* structural flagella antigen H7 ($fliC_{H7}$) per Wang et al. (2002); and 3) for intimin specific for *E. coli* O157:H7 ($eaeA_{O157}$) per Gannon et al. (1997). All reactions included a primer set for the 16S rRNA gene for *E. coli* to control to serve as an internal control per Wang et al. (2002).

Following cycler programs (iCycler, Bio-Rad, Hercules, CA), 2µl of sample and 1µl 10xbluejuice (Invitrogen, Carlsbad, CA) was added to a 100 ml 1xTBE buffer:1g agarose (Certified PCR Agarose, Bio-Rad, Hercules, CA) gel well, submerged in 80 ml 1xTBE buffer. A 20 well comb was used to cast each PCR gel (15 samples, 3 lambda ladder wells, one positive control, and one negative control). After electrophoresis (1 hr at 80v), gels were removed from buffer stained for 30 min with ethidium bromide, and de-stained for 20 min. A Gel Doc EQ (Bio-Rad, Hercules, CA) was used for ultra-violet photo imaging,xported gel images were analyzed using the QuantityOne (Bio-Rad, Hercules, CA) software program.

Pulsed-Field Gel Electrophoresis

Isolate cells at a 3.0 McFraland (Remel Colormeter Standards kit, Lenexa, KS) turbidity were suspended in Cell Suspension Buffer (CSB; 1.0 M NaCl, 10 ml 1.0 M Tris [Sigma, Desienhofen, Germany], pH 7.6) then added in a 1:1 ratio to 1.6% Low Melt Agarose (Bio-Rad, Hercules, CA) and CSB solution and set into individual plugs (10mm x 5mm x 1.5mm). Cells in plugs were then digested using 0.5 M EDTA (Bio-Rad, Hercules, CA), 1% Sodium Lauroyl Sarcosine (Sigma, Desienhofen, Germany),

and 0.5mg Proteinase K (Sigma, Desienhofen, Germany). After digestion, buffer was removed and plugs were washed/incubated (37°C for 30 min) 5 times with 5 ml TE buffer (10 mM Tris [Bio-Rad, Herculs, CA], 0.1 mM EDTA [Bio-Rad, Hercules, CA]).

Digested plugs, 400µl sterile H_20, and 40µl 10X reaction buffer (Roche, Indianapolis, IN) were incubated, (4°C) overnight. Reaction buffer solution was removed/replaced, and incubation was allowed to continue, (24 ± 2 °C) for 4 h. After incubation, 6µl of *Xba*I (Roche Molecular Biochemicals, Indianapolis, IN) restriction enzyme was added to each centrifuge tube and incubated in a dry incubator, (37°C) overnight, then stopped by removing enzyme solution and adding enough TE buffer to cover the restricted plug followed by incubation, (37°C) for 1 hr.

Restricted plugs were inserted into solidified 1% Pulsed Field Certified Agarose (Bio-Rad, Hercules, CA) and 0.5X TBE buffer (Tris-Borate EDTA Buffer [Sigma, St. Louis, MO]) gel wells, then placed into 2L of 0.5X TBE buffer at 14°C in the electrophoresis chamber of the CHEF Mapper PFGE System (Bio-Rad, Hercules, CA) with the following electrophoresis parameters: 1) gradient 6.6 V/cm; 2) 120° Angle; 3) initial switch time of 2.16 sec; 4) final switch time of 54.17 sec; 5) linear ramp factor; and, 6) run time of 20 hours. Following ethidium bromide stain/de-staining, gels were placed in Gel Doc EQ (Bio-Rad, Hercules, CA) for ultra-violet photo imaging. Exported gel images were analyzed by the FingerPrinting II software program (Bio-Rad, Hercules, CA).

Results and Discussion

For all sample locations, with the exception of restrainer side walls and feed samples, *E. coli* O157:H7 was detected.

Multiplex-PCR

Using three Multiplex-PCR protocols (Gannon et al., 1996; Wang et al., 2002), all isolates were examined for known *E. coli* O157 gene targets identifying isolates that posses the capability to produce Shiga Toxins 1 and 2 (*Stx*1) and (*Stx*2), enterohemorrhagic *E. coli* hemolysin (EHEC *hylA*), the gene which encodes for the *E. coli* O157 serotype ($rfbE_{0157}$) (Wang et al., 2002), the *E. coli* flagellum H7 serotype ($fliC_{H7}$), and the $eaeA_{O157}$ gene which is responsible for the production of intimin and is involved in attaching and effacing adherence (Gannon et al., 1996; Wang et al., 2002).

At least one isolate recovered from the feedlot pen floors, feedbunks, loading chutes, transport trailers, processing plant holding pen railings and water tanks, possessed at least one of the *Stx*1, *Stx*2, *hyl*A, $rfbE_{O157}$, $fliC_{H7}$, and $eaeA_{O157}$ genes (Table 1). Feedlot water and processing facility holding pen floor isolates possessed all genes screened for, with the exception of the gene for *Stx*2 production. Interestingly, over 50.0% of loading chute, transport trailer walls, holding pen railing and holding pen water derived isolates had all six genes present (Table 1).

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was used to further evaluate the genetic relatedness of *E. coli* O157:H7 isolates recovered from hides, as well as from associated colon and companion samples. Isolate banding patterns were first separated

into factions of isolates related to the three processing facilities, and further separated into clusters of isolates that produced the same virulence gene profiles. Once environmentally linked isolates with identical gene marker profiles were grouped, individual band patterns were compared to the other patterns in that cluster. Since isolates were collected within a one year interval, and were restricted using a single restriction endonuclease, the method as described by Tenover et al. (1995) was used to analyze the isolate clusters. Tenover et al. (1995) suggests the following relationship classification when the band patterns of two isolates are compared: 0-1 band differences are considered genetically indistinguishable, 2-3 band differences are considered probably or closely related, 4-6 band differences are considered possibly related, and compared patterns with ≥7 band differences should be considered genetically different from one another. Indistinguishable relationships (0-1 differences) were expressed between two isolates derived from colons of animals from the same feedlot pen, and between two isolates of feedlot and plant holding pen origin (Table 2). Probable genetic relationships (2-3 differences) were observed between isolates derived from: hide and colon samples (cross-contamination); colons and feedlot locations; colons and transport trailers; and feedlot and plant receiving locations (Table 2). Possible genetic relationships (4-6 differences) were observed between: hide-derived isolates and feedlot, transport trailer and plant holding facility locations, as well as from other hides and colons (cross-contamination); colon-derived isolates and feedlot and transport trailer locations, as well as other hides and colons; different pens at the same feedlot; feedlot and plant holding pen locations; and plant holding and transport trailer locations (Table 2). As shown (Table 2), relationships were expressed between isolates cultured from samples that, upon initial analysis, did not have an epidemiological association (i.e. a relationship between isolates cultured from feedlot pen samples and transport trailer samples). However, matching bands do not always contain homologous genetic material (Davis et al., 2003), given that it is possible for genetically different restriction fragments to travel similar distances. There were also proposed relationships (2, 4, and 5 band differences, respectively) between 3 pairs of feedlot-derived isolates when compared to plant holding pen isolates. Although this improbable relationship could also be explained by non-homologous fragments at the same molecular weight distance, a number of these cattle feeding facilities used packer-owned transport trailers to haul cattle to the packing facility. These trucks haul cattle from similar feeders continually during the year, and there is potential for a dominant feedlot *E. coli* O157:H7 strain to establish itself in a plant holding pen environment if a sanitary atmosphere is not maintained.

Documented trailer condition ranged from new to excessively dirty; therefore, four categories of trailer condition were created. “Very Clean” (new, to adequately washed and dry, with no visible organic material present); “Moderately Clean” (poorly washed with little or no dry organic matter on floors and walls); “Moderately Unclean” (1-2 previous loads post-wash, excessive dry, or moderate wet organic matter present on floors and walls); and “Very Unclean” (3+ loads post-wash, with excessive amounts of wet organic matter present on trailer walls/floors). A total of 15 isolates were recovered from 7 (38.89%) of the 18 trailers. These 7 *E. coli* O157:H7 positive trailer samples were collected from 1 (14.29%) “Very Clean” trailer, 4 (57.14%) “Moderately Clean” trailers, 1 (14.29%) “Moderately Unclean” trailer, and 1 (14.29%) “Very Unclean” trailer, respectively. In that, 71.43% of the trailers from which *E. coli* O157:H7 isolates were cultured were considered “Very Clean” or “Moderately Clean”; this data should lead to the further investigation of current trailer cleaning and sanitation practices. A plausible explanation for the large number of

clean trailers that tested positive for pathogenic contamination could be the lack of competitive organisms found at the clean trailer sample site or possible sample contamination.

Conclusions

Escherichia coli O157:H7 isolates expressing the genes for virulence factors such as Shiga toxin production (*Stx*1, *Stx*2), known to cause the symptoms of Hemolytic Uremic Syndrome, the Intimin gene involved in adhering and effacement of the organism (*eae*A_{O157}), and the virulent toxin enterohemolysin (*hyl*A_{O157}) were recovered from feedlot pen, loading chute, transport trailer, and packing plant holding facility sample types, as well as from cattle hides and colons at the time of slaughter. In this research, genetic relationships (Tenover et al., 1995) were expressed between hide derived *E. coli* O157:H7 isolates and isolates recovered from feedlot pen floors, feedlots pen water tanks, feedlot feedbunks, loading chutes, transport trailers, packing plant holding pens, holding pen water tanks, and from the hides and colons of other cattle.

In that, *E. coli* O157:H7 isolates with pathogenic capabilities were recovered from locations which also resulted in the transfer of similar organisms onto the hides of slaughter ready cattle, it is imperative that the feedlot and loading chute, transportation trailers and packing plant holding areas are all considered likely reservoirs of pathogenic *E. coli* O157:H7 organisms, which can result in hide contamination that may easily lead to contamination of beef carcasses subsequent and beef products.

Additionally, current methods used to wash cattle trailers after shipment may not be effective, as the largest numbers of isolates were recovered from trailers characterized as Very Clean and Moderately Clean. Existing cleaning protocols may need to be followed by a sanitation method to effectively remove pathogenic contamination from the walls and floors of trailers.

Table 1. Distribution of *E. coli* O157:H7 virulence genes (eaeA, Stx1/2, hylA, rfbE, fliC) among each sample type.

Feedlot	# of Isolates	*eae*A$_{O157}$	*Stx*1	*Stx*2	*hyl*A	*rfb*E	*fli*C	All
Water	3	66.7 (2/3)	66.7 (2/3)	0.0(0/3)	100.0(3/3)	100.0(3/3)	100.0 (3/3)	0
Pen floor	45	93.5(43/46)	65.2(30/46)	52.2(24/46)	95.7(44/46)	93.3(42/46)	93.5(43/46)	9
Feed	nd							0
Feedbunk	2	50.0(1/2)	50.0(1/2)	50.0(1/2)	50.0(1/2)	50.0(1/2)	50.0(1/2)	0
Loading chute	14	92.9(13/14)	50.0(7/14)	64.3(9/14)	92.9(13/14)	92.9(13/14)	92.9(13/14)	6
Transport								
Trailer walls	14	92.9(13/14)	57.1(8/14)	85.7(12/14)	92.9(13/14)	92.9(13/14)	92.9(13/14)	7
Trailer floor	5	60.0(3/5)	40.0(2/5)	60.0(3/5)	40.0(2/5)	60.0(3/5)	40.0(2/5)	1
Processing facility								
Pen floor	3	33.3(1/3)	33.3(1/3)	0.0(0/3)	33.3(1/3)	100.0(3/3)	33.3(1/3)	0
Pen side rails	8	87.5(7/8)	75.0(6/8)	75.0(6/8)	75.0(6/8)	100.0(8/8)	75.0(6/8)	6
Water	5	100.0(5/5)	80.0(4/5)	80.0(4/5)	100.0(5/5)	100.0(5/5)	100.0(5/5)	4
Restrainer	nd							0
Carcasses								
Hide	176	58.4(101/173)	34.1(59/173)	38.7(67/173)	50.9(88/173)	78.6(136/173)	53.2(92/173)	24
Colon	48	93.8(45/48)	47.9(23/48)	35.4(17/48)	83.3(40/48)	87.5(42/48)	83.3(40/48)	3
Totals	**322**							**60**

Table 2. Comparison of relationships between restriction band pattern differences of isolates recovered from different samples from the same collection lot, expressing similar genotypes (Multiplex-PCR). Relationships were categorized into four categories; Indistinguishable (0-1 differences), Probably Related (2-3 differences), Possibly Related (4-6 differences) and Different (Tenover et al., 1995).

Sample Type	Epidemiological Relationship (N of Relationships)		
	Indistinguishable	Probably Related	Possibly Related
Hide/Hide		X (3)	X (7)
Hide/Colon			X (1)
Hide/Feedlot pen floor		X (2)	X (3)
Hide/Feedlot pen water			X (1)
Hide/Trailer			X (2)
Hide/Plant pen water			X (1)
Colon/Colon	X (2)		X (2)
Colon/Feedlot pen floor		X (2)	X (5)
Colon/Trailer		X (2)	X (1)
Loading chute/Plant pen walls		X (1)	X (1)
Feedlot pen floor/Plant pen walls			X (1)
Feedlot pen floor/Plant pen floor	X (1)	X (2)	
Loading chute/Trailer			X (1)
Plant pen floor/Trailer			X (1)
Feedlot pen/Feedlot pen (same feedlot, different pens)			X (2)