Non-O157 Shiga Toxin-producing *E. coli*: Status and Relevance to Food Safety

The Food Safety Group
U.S. Meat Animal Research Center
USDA-ARS
Clay Center, Nebraska

Presentation Outline/Objectives

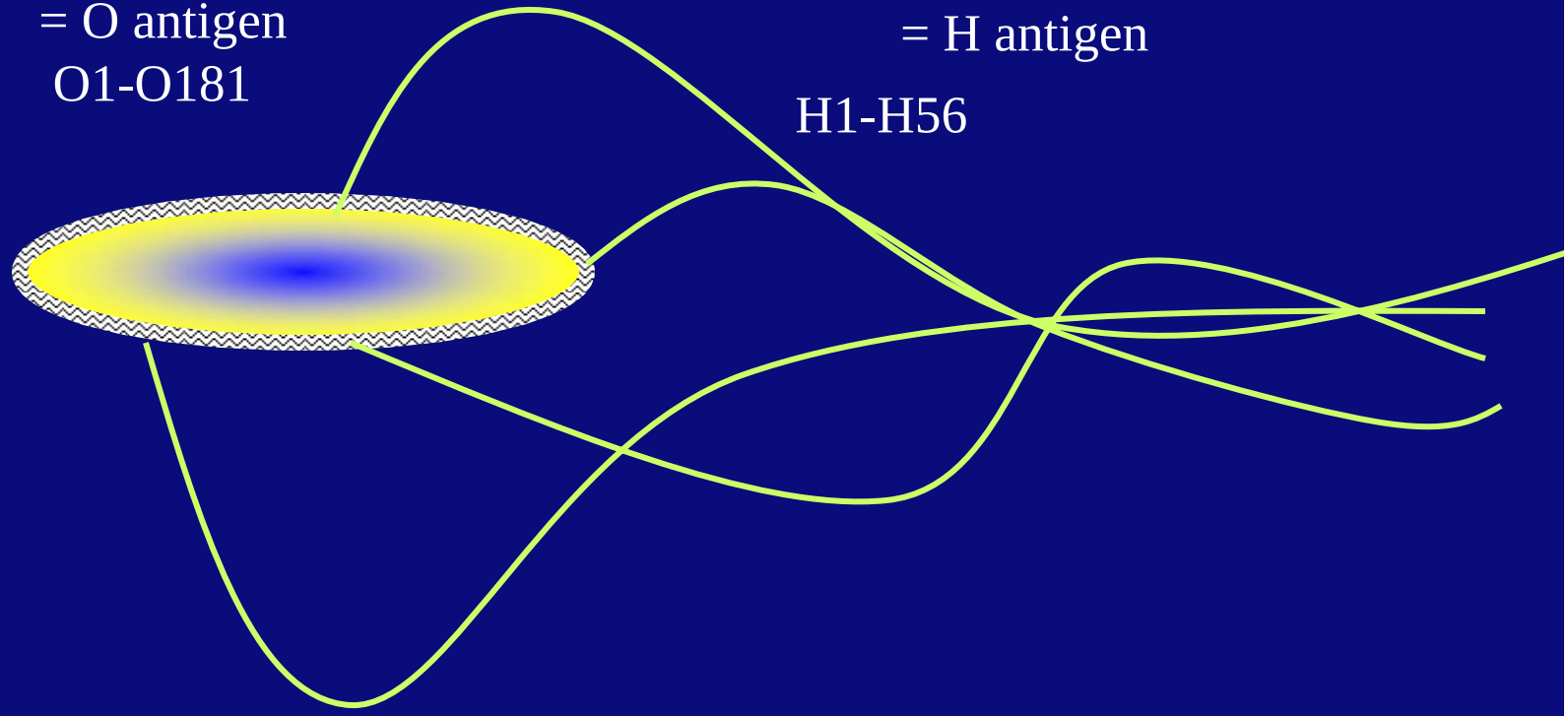
- Introduction
- Our perspective on non-O157 STEC
- Prevalence of non-O157 STEC
- Efficacy of the current interventions
- Summary and concluding remarks

Nomenclature

E. coli serotyping

Lipopolysaccharide (LPS)
= O antigen
O1-O181

= H antigen
H1-H56



O26:H11

Our Perspective

- Mode of Operation (for any pathogen)
  - What is the prevalence?
  - Are the current interventions effective?
  - What is the prevalence in the ground beef supply – should we be concerned?

Our Perspective

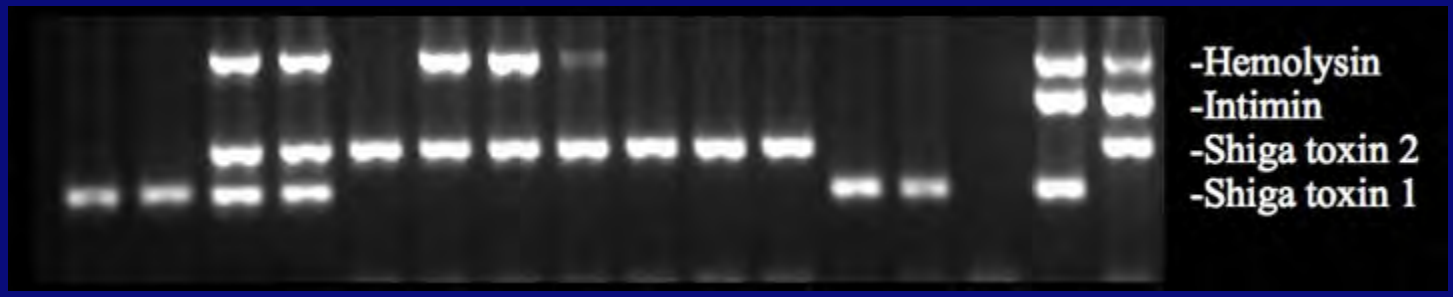
- Although non-O157 STEC is getting a lot of media attention recently, this is not a new issue for us; we have been working on this issue for years - collecting and publishing data, as well as testing interventions that will reduce non-O157 in meat products.
- There are many kinds of non-O157 STEC, but only a subset appears to be important for human disease.

Our Perspective

- STEC are a natural part of the animal microflora.
- The interventions that work to reduce STEC O157 on meat also work to reduce non-O157 STEC.
- Finding non-O157 STEC is not easy, but we have made progress in developing methods that work, and we are happy to share them.

Methodology (until 2006)

- Prepare samples as with *E. coli* O157:H7
- Enrich as with *E. coli* O157:H7
- PCR a sample of the enrichment for Shiga toxin genes

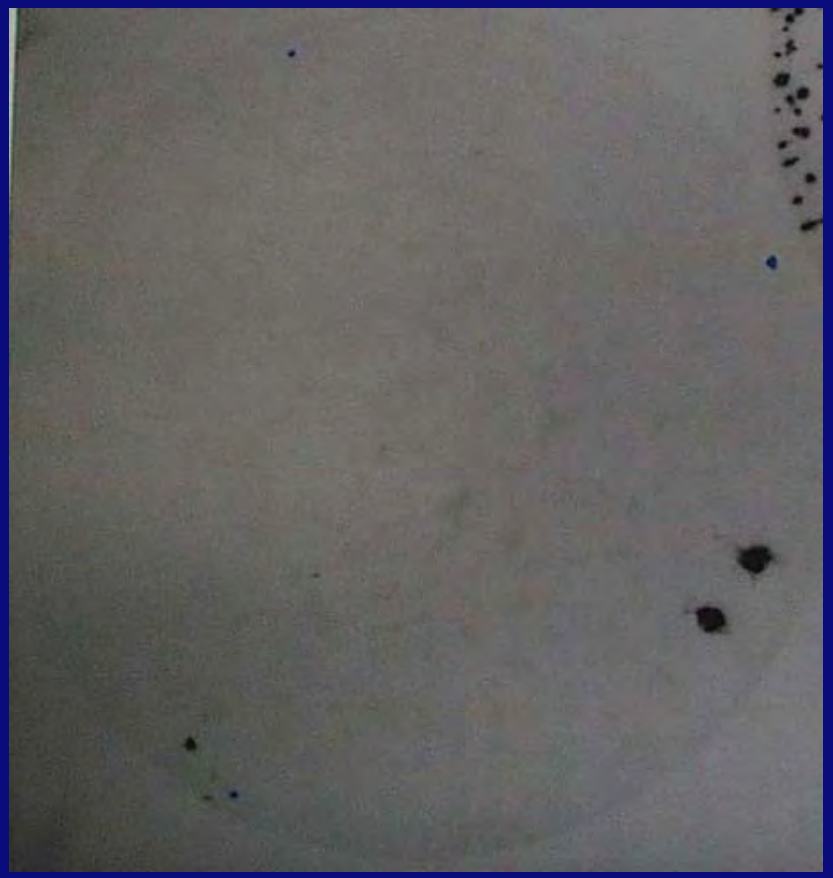


Methodology (until 2006)

Colony Hybridization

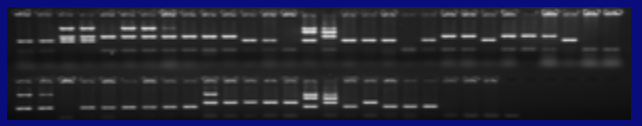
- Grow colonies from sample enrichments on agar media
- Transfer colonies to nylon membranes
- Lyse cells and fix DNA to the membrane
- Hybridize with DNA probes for Shiga toxin genes
- Detect bound probe
- Identify target colonies


WHEATON



Methodology - Continued

- Pick colony and obtain pure culture for characterization
- Characterize for virulence factors



- Perform biochemical characterization to confirm that isolates are *E. coli*
  - *Shigella dysenteriae*, *Citrobacter freundii*, and *Enterobacter cloacae* have been found to produce Shiga toxins.
- Once confirmed, then serotype (O and H typing)

Washed Sheep Blood Agar
for isolation of non-O157 STEC

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NOTES

Rapid Detection and Isolation of Shiga-Like Toxin (Verocytotoxin)-Producing *Escherichia coli* by Direct Testing of Individual Enterohemolytic Colonies from Washed Sheep Blood Agar Plates in the VTEC-RPLA Assay

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By combining the enterohemolysin test and the VTEC-RPLA test (specific for the detection of Shiga-like toxin I [SLT-I], SLT-II, and SLT-IIc), single colonies of SLT-producing *Escherichia coli* were found to constitute between 0.03 and 68.1% of the coliform flora in human stool cultures and were isolated and characterized within 72 to 96 h.

Some types of Shiga-like toxin (SLT)-producing *Escherichia coli* (SLTEC) are important human pathogens causing hemorrhagic colitis and hemolytic uremic syndrome (HUS). The detection of these pathogens from patients' stool samples can be complicated when SLTEC strains are present in low numbers and also if phenotypical traits for their identification are absent (4, 8). About 90% of SLTEC strains isolated from humans exhibit a typical enterohemolytic phenotype on washed sheep blood agar which can be employed as a diagnostic marker for their identification (1, 3). In order to establish a rapid identification and isolation system for SLTEC from human stool, we combined the enterohemolysin test as a microbiological screening system for SLTEC with the VTEC-RPLA test as a rapid detection system for SLT production.

The SLTEC strains and the *E. coli* hemolysins are described elsewhere (2, 3, 7). Washed sheep blood agar plates were prepared in our laboratory (3) and compared with commercially available enterohemolysin agar plates (9) for the detection of different *E. coli* hemolysin (3, 7). No difference between both types of plates for detection of different hemolytic phenotypes was found. All SLTEC strains were analyzed for Vero cell toxicity as described previously (3, 5). *slt I* and *slt II* genes were detected by DNA-DNA hybridization and by *slt I*-, *slt II*-, and *slt IIc*-specific PCR (2, 3). *slt II* and *slt IIc* were distinguished as described previously (6).

For the isolation of fecal SLTEC, a small amount of stool was inoculated into a tube containing 5 ml of sterile tryptic soy broth. The stool culture was incubated without shaking for 20 to 22 h at 37°C. The next day, the grown culture was serially diluted 10-fold in phosphate-buffered saline (PBS), pH 7.2. From each dilution (10^{-5} to 10^{-7}), 0.1 ml was spread with a glass rod on Endo agar (Merck, Darmstadt, Germany) and enterohemolysin agar. The plates were incubated for 20 to 22 h at 37°C. The enterohemolysin agar plates were recorded for hemolysis after 3 h of incubation (indicating only α-hemolysin) and after overnight incubation (indicating all types of hemolysins). The titer of coliform bacteria was calculated by counting

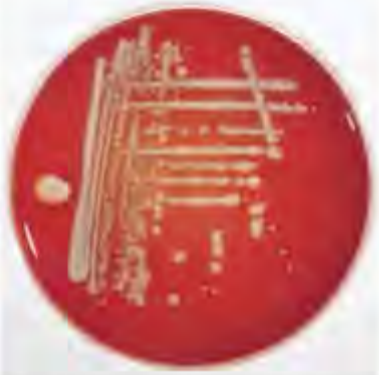

FIG. 1. Enterohemolysin agar plate inoculated with the stool culture of patient 6 (Table 2) after incubation for 22 h at 37°C. Colonies of *E. coli* O157:H7 become visible by their enterohemolytic phenotype after overnight incubation.

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Mitomycin-supplemented washed blood agar for the isolation of Shiga toxin-producing *Escherichia coli* other than O157:H7

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K. SUGIYAMA, K. INOUE AND R. SAKAZAKI: 2001

Aims: Isolation and recognition of the prominent Shiga toxin (Stx)-producing strains of *Escherichia coli* (STEC) serovar O157:H7 can be confirmed easily by their late fermentation of sorbitol and lack of β-glucuronidase activity, but there has been no culture method of choice for detecting non-O157 STEC strains because of their biochemical diversity. Apart from Stx, many STEC strains produce enterohaemolysin (Ehly) regardless of their serovars.

Methods and Results: Although washed blood agar media, with or without the addition of antibiotics (vancomycin, cefixime, and cefsulodin) (WBA and WBVCCA), have been used to detect Ehly, a proportion of STEC strains consistently failed to produce haemolysin on these media. Washed blood agar medium was therefore studied further in order to increase the yield of strains producing Ehly.

Conclusions: It was found that the addition of 0·5 μg ml^{-1} of mitomycin C to the agar medium (WBMA) markedly increased the number of such strains. Thus, of 185 STEC strains comprising 95 O157 and 90 non-O157 STEC consisting of 34 serovars. Ninety-seven per cent of these strains produced haemolysis on WBMA, compared with only 76% and 83%, respectively, on WBA and WBVCCA.

Significance and Impact of the Study: The appearance of the Ehly zone of haemolysis that was easily distinguishable from that of α-haemolysin was enhanced by the incorporation of mitimycin C into washed-blood medium.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is an important human pathogen causing haemorrhagic colitis and the haemolytic uraemic syndrome (HUS). The most important serovar implicated in these conditions is *E. coli* O157:H7, and selective media containing sorbitol or a chromogenic substrate for β-glucuronidase activity are used in the routine screening for this serovar or its nonmotile variant. STEC serovars other than O157:H7 have also been implicated, both in sporadic cases and in outbreaks. However, no biochemical markers have been found to distinguish them from commensal *E. coli*. The only cultural method for the detection of non-O157 STEC strains uses washed sheep blood agar containing Ca^{2+} ions (WBA) (Beutin *et al.* 1989). The method is based on the finding that STEC strains produce a characteristic zone of haemolysis on this agar. Unfortunately, a considerable proportion of non-O157 STEC strains failed to produce Ehly on this medium (Beutin *et al.* 1989; Bettelheim 1995).

Law *et al.* (1992) described the detection by ELISA of low numbers of STEC in mixed cultures grown in the presence of mitomycin C. We added mitomycin C to WBA (WBMA) in expectation of the same effect on Ehly production and found a marked increase in the number of Ehly-positive strains. WBMA was also compared with an another agar-based medium (WBVCCA) developed by Lehmacher *et al.* (1998), who added antibiotics to WBA to give some selectivity for STEC strains. In the present paper, the

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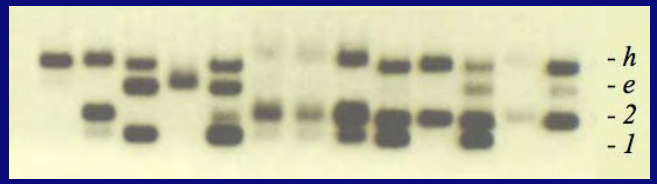
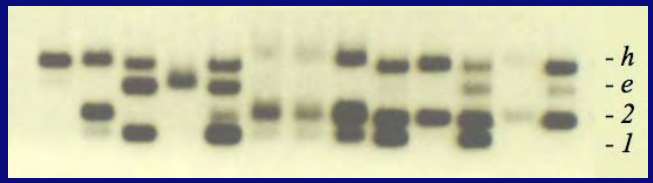
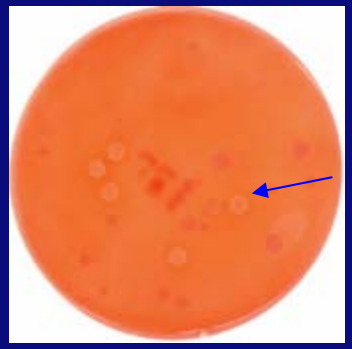
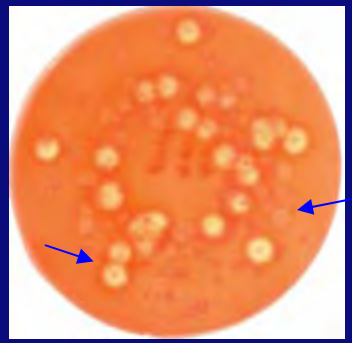
Colony Hybridization



Sheep Blood Agar







Complexity of the current non-O157 Assay

- 0 Hr: Sample arrives, is weighed and TSB is added for enrichment for 12 hrs at 42^{o}C
- At 12th hr: A sample is removed for detection of virulence factors by PCR – takes 3-4 hrs
- At the 16th hr: If positive, a sample of the enrichment is plated onto sheep blood agar and allowed to grow at 37^{o}C for 16-18 hrs
- At the 34th hr: Colonies are picked for virulence factor detection again – 3-4 hrs
- At the 38th hr: Streak onto MacConkey agar and incubate overnight
- At 50th hr: Pick a colony, make an agar stab and ship for serotyping - takes a week to two to get the results back

Best case: 62 continuous hrs; reality: 2 weeks

Top Non-O157 Serotypes (CDC)

- O26 22% of non-O157 STEC
- O111 16% of non-O157 STEC
- O103 12% of non-O157 STEC
- O121 9% of non-O157 STEC
- O45 7% of non-O157 STEC
- O145 5% of non-O157 STEC

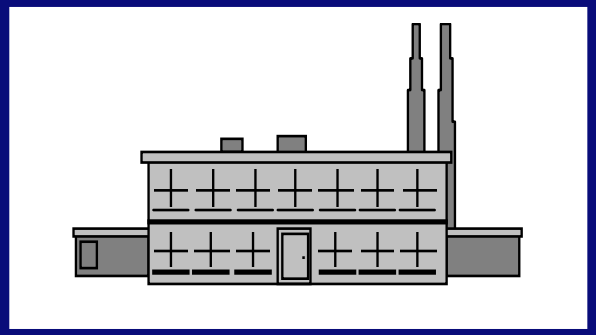
Prevalence of Non-O157 STEC

- Commercial fed cattle processing plants
- Commercial fed cattle processing plants as a function of the season of the year
- Commercial cow/bull processing plants
- Commercial lamb processing plants
- Imported raw ground beef material (trim)
- National ground beef supply

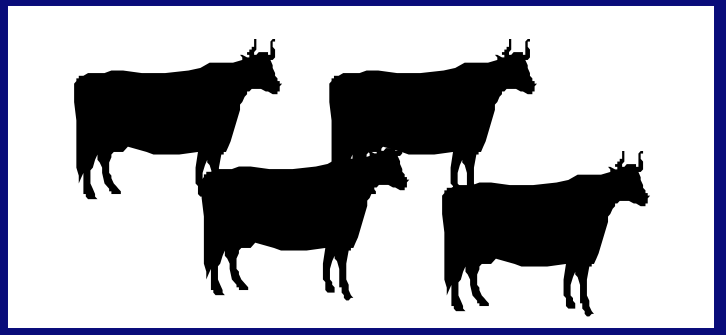
We are very appreciative of the U.S. meat industry for allowing us to use their facilities as our laboratory.

Commercial Fed Cattle Processing Plants

E. coli O157:H7/NM in-plant study

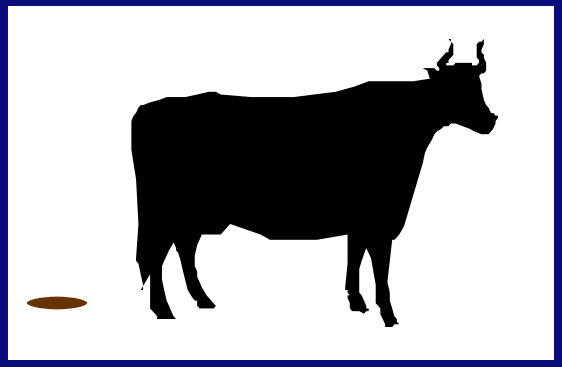


4 large packing plants,
two trips each

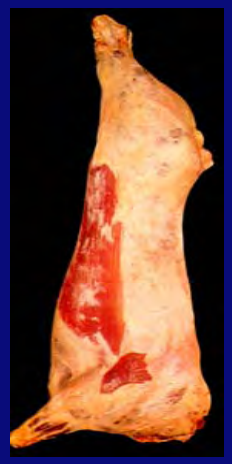


3-4 lots of 35-85
animals each trip

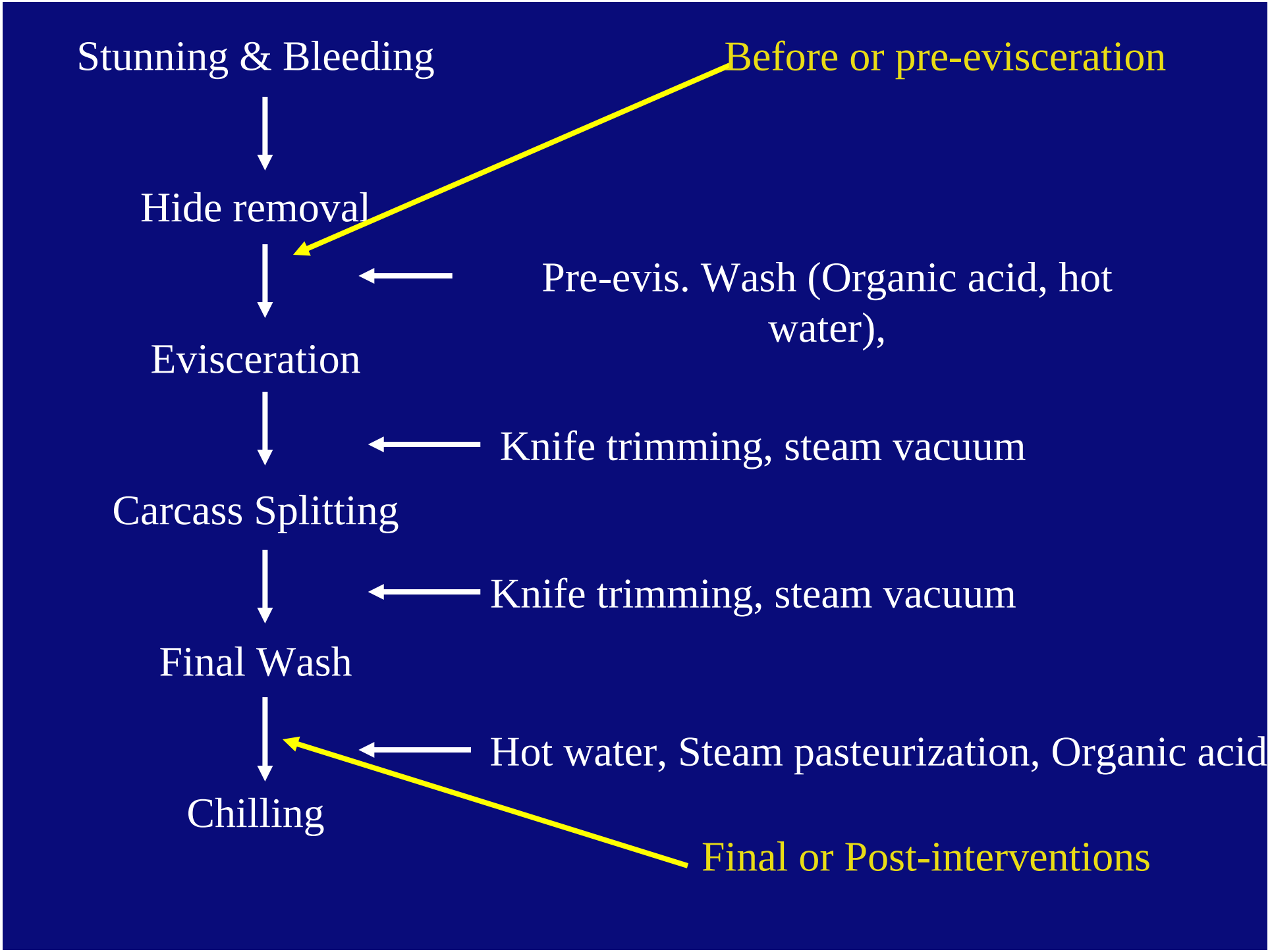
Sample 20% of each lot:



Preharvest: hides, feces



Postharvest (tracked carcasses):
preevisceration, postevisceration, and after final interventions (in the cooler)


Stunning & Bleeding
Before or pre-evisceration
Hide removal
Pre-evis. Wash (Organic acid, hot water),
Evisceration
Knife trimming, steam vacuum
Carcass Splitting
Knife trimming, steam vacuum
Final Wash
Hot water, Steam pasteurization, Organic acid
Chilling
Final or Post-interventions

Results

	Pre-evisceration (No intervention)	Final (after all interventions)
E. coli O157	44.4% (144/324 carcasses)	1.8% (6/326 carcasses)
Non-O157 STEC	54% (180/334 carcasses)	8.3% (27/326 carcasses)

Serogroup Distribution of Non-O157 STEC Isolates

Serogroup	# of isolates	Before	Final
O142	54	46	8
→ O121	31	31	0
O2	22	19	3
O171	18	18	0
O113	15	12	3
O132	14	13	1
O8	13	11	2
O88	10	10	0
O6	9	8	1
O139	9	5	4
O172	9	7	2
OX3	9	3	6
O104	5	1	4
O117	5	5	0
O15	4	4	0
O165	4	4	0
O3	3	3	0
O55	3	3	0
O153	3	3	0
O168	3	0	3
O10	2	2	0
→ O45	2	2	0
→ O103	2	2	0
O109	2	0	2
O119	2	2	0
→ O145	2	2	0
OX25	2	2	0

None of the top 6 CDC serotypes were found on the carcasses after the full complement of all the interventions:

- Interventions are effective

Virulence Attributes

• *E. coli* can cause human disease when they possess *stx*1 or *stx*2.

• Individuals infected with strains producing Shiga toxin 2 are more likely to develop severe disease than those infected with strains carrying Shiga toxin 1.

• It is commonly thought that *E. coli* must contain *stx*1 or *stx*2 and *eae* (intimin) to have the highest chance of causing disease in humans – of course there are always exceptions.

STEC Virulence Factor Profiles

STEC virulence factors	# of Isolates	Before	Final
*stx*1	152	135	17
*stx*2	93	78	15
*stx*1, *stx*2	15	15	0
*stx*1, *eae*	2	2	0
*stx*1, *hlyA*	8	3	5
*stx*2, *hlyA*	19	17	2
*stx*1, *stx*2, *hlyA*	31	23	8
*stx*1, *stx*2, *eae*	1	1	0
*stx*1, *eae*, *hlyA*	8	6	2
*stx*2, *eae*, *hlyA*	20	20	0
*stx*1, *stx*2, *eae*, *hlyA*	12	10	2
Total	361	310	51

From 2/326 carcasses

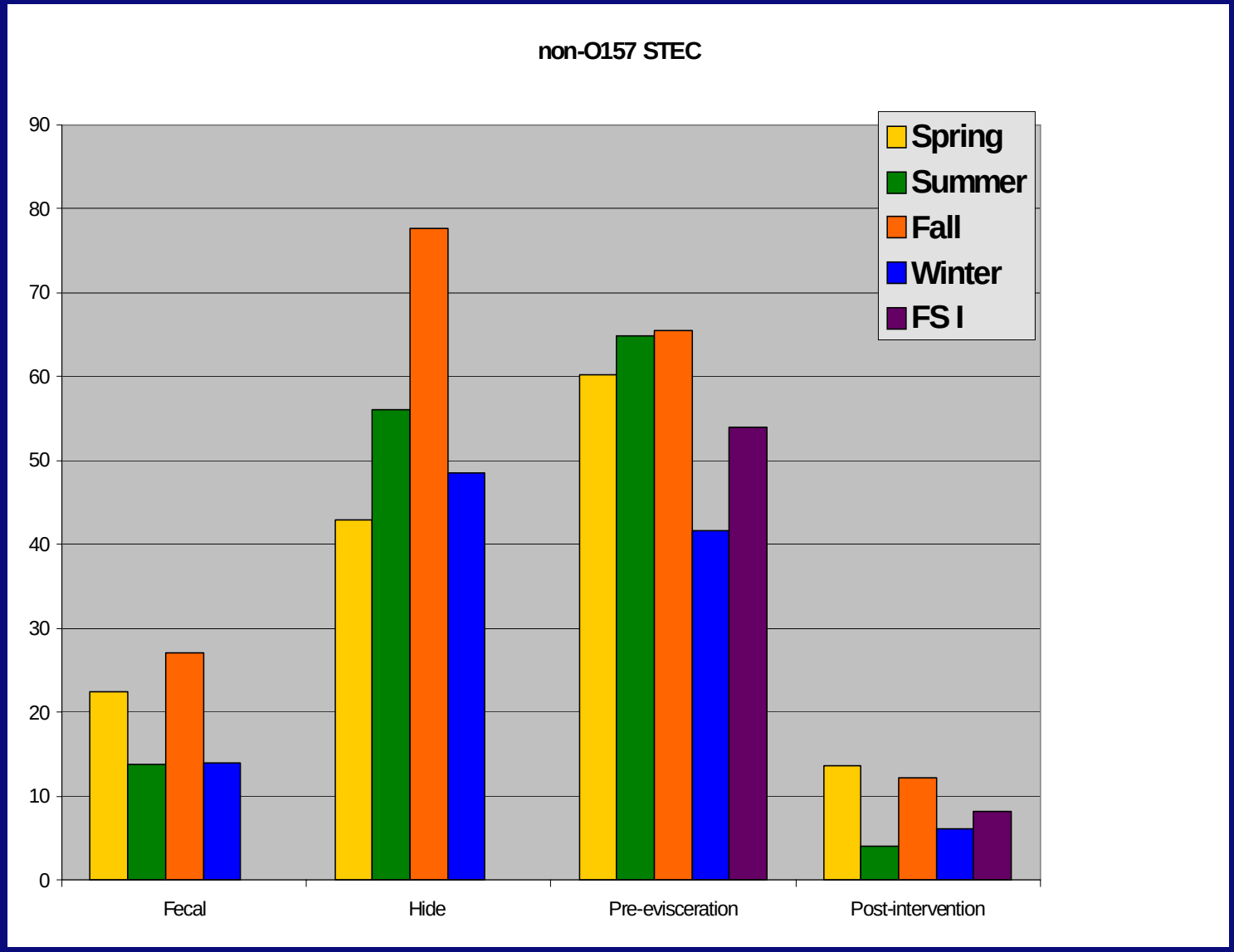
Before & After = Before and after interventions

Prevalence of Non-O157 STEC

- Commercial fed cattle processing plants
- Commercial fed cattle processing plants as a function of the season of the year.
- Commercial cow/bull processing plants
- Commercial lamb processing plants
- Imported raw ground beef material (trim)
- National ground beef supply

Study Design

- Season effect
- *E. coli* O157, *Salmonella*, non-O157 STEC
- 3 plants
- 2 visits/plant/season
- 100 samples/site/plant/season
- Feces, hide, pre-evisceration, and post-intervention samples came from the same animal/carcass


non-O157 STEC
90
80
70
60
50
40
30
20
10
0
Fecal
Hide
Pre-evisceration
Post-intervention
Spring
Summer
Fall
Winter
FS I

STEC Virulence Factor Profiles

Virulence factors	Fecal	Hide	Before	After
*stx*1	66	678	298	64
*stx*2	187	223	657	83
*stx*1, *stx*2	31	39	98	12
*stx*1, *hlyA*	49	71	46	12
*stx*2, *hlyA*	93	152	211	20
*stx*1, *stx*2, *hlyA*	39	52	125	9
*stx*1, *eae*	1	1	1	0
*stx*2, *eae*	3	3	1	0
*stx*1, *stx*2, *eae*	0	0	1	0
*stx*1, *eae*, *hlyA*	32	34	62	19
*stx*2, *eae*, *hlyA*	17	31	19	11
*stx*1, *stx*2, *eae*, *hlyA*	0	10	8	9
Total	518	1294	1527	239

From 22/1232 carcasses

Enumeration of STEC on Post-Intervention Carcasses
(as determined by PCR for *stx*)

Cells per 100 cm^2

Season	# Samples	MPN Index	95% C.I.
spring	66	< 3.0	0.0-9.5
spring	1	3.6	0.2-18.1
spring	1	7.4	1.3-20.3
spring	1	38.2	17.7-82.6
summer	32	< 3.0	0.0-9.5
fall	63	< 3.0	0.0-9.5
fall	2	3	0.2-9.6
fall	3	3.6	0.2-18.1
winter	31	< 3.0	0.0-9.5

Prevalence of Non-O157 STEC

- Commercial fed cattle processing plants
- Commercial fed cattle processing plants as a function of the season of the year.
- Commercial cow/bull processing plants
- Commercial lamb processing plants
- Imported raw ground beef material (trim)
- National ground beef supply

Study Design

- 3 plants
- Samples collected in spring/summer
- 3 days of sample collection
- 96 samples/site
- Pelt/fleece, Pre-evisceration, and Post-intervention
- APC, *E. coli* O157:H7, *Salmonella*, and non-O157 STEC

Prevalence of Non-O157:H7 STEC at Different Sites in Lamb Processing Plants (*stx* PCR)

		# Positive (%)		
	N	Pelt	Before	Final
Stx PCR	846	729 (86.2)	665 (78.6)	690 (81.6)
Isolate	846	-	-	488 (57.7)

STEC Virulence Factor Profiles

STEC virulence factors	# of isolates	% of isolates
*stx*1	91	18.6
*stx*2	9	1.8
*stx*1, *stx*2	224	46.0
*stx*1, *hlyA*	19	3.9
stx2, *hlyA*	1	0.2
*stx*1, *stx*2, *hlyA*	142	29.1
*stx*1, *eae*	0	0.0
*stx*2, *eae*	0	0.0
*stx*1, *stx*2, *eae*	0	0.0
*stx*1, *eae*, *hlyA*	2	0.4
*stx*2, *eae*, *hlyA*	0	0.0
*stx*1, *stx*2, *eae*, *hlyA*	0	0.0
Total	488	100.0

Non-O157:H7 STEC Found on Post-Intervention Lamb Carcasses

STEC	# isolates	STEC	# isolates
OUT:H2	3	O91:H14	149
OUT:H2/35	5	O103:H38	2
OUT:H3	2	O109:H30	2
OUT:H10	9	O128:H2	3
OUT:H12	3	O128:H2/35	64
OUT:H14	2	O128:H3	4
O5:H19	90	O146:H8	11
O6:H10	7	*O146:H21*	1
O8:H9	3	O146:H36	3
O15:H27	5	O174:H8	9
O36:H7	4	O169:H19	1
O76:H19	7	OX18:H36	7
		Others	36

Highlight as pink = asso.w/ HUS; Underline = asso. w/ cattle; Italic and yellow = human STEC

None are on the CDC top 6 list

STEC Prevalence in Imported and Domestic Boneless Beef Trim Used for Ground Beef

Samples for analysis were supplied by 2 large importers of boneless beef trim.

Australia
n = 220

New Zealand
n = 223

Uruguay
n = 256

Domestic (U.S.)
n = 487

STEC

Frequency of STEC isolation

in boneless beef trim by country of origin

	AUS	NZ	URU	DOM
n	220	223	256	487
Isolate positive samples	9	4	40	28
STEC isolated	10	4	52	32

Serotypes of STEC isolated by country

AUS	NZ	URU		DOM	
O33:H11	O26:H8	O2:H25	O116:H36	O5:H36	O117:H+
O73:H35	**O26:H11**	O6:H30	O130:H11	**O8:H19**^{x3}	O132:H+
O113:H36^{x3}	O64:H9	O6:H34	**O163:H19**^{x3}	**O20:H19**	O132:H38
O113:H51	**O163:H19**	O8:H3	O163:H26	O55/83:H15	O142:H34
O147:H7		**O8:H19**^{x2}	**O168:H+**	O73:H+	O150:H2/35
O171:H+		O15:H27^{x3}	O174:H11	O73:H18	**O165:H-**
ONT:H+		**O20:H19**^{x4}	O174:H28^{x2}	**O79:H7**	O171:H2
ONT:H2		O39:H14	O174:H36^{x2}	O83:H+	O172:H10
		O55/83:H15	ONT:H+^{x2}	O83:H38	O174:H36
		O74:H28^{x2}	**ONT:H11**^{x2}	O83/132:H2	OX25:H11
		O82:H8	ONT:H18	O88:H38	ONT:H2
		O82:H15	ONT:H19^{x2}	O113:H4	ONT:H7
		O83:H8^{x2}	ONT:H32	O113:H51	ONT:H32
		O83:H11	ONT:H34	O116:H21	ONT:H51
		O88:H38^{x2}	ONT:H46^{x4}		**OR:H-**
		O113:H21	ONT:H51		
		O113:H36	ONT:H52		

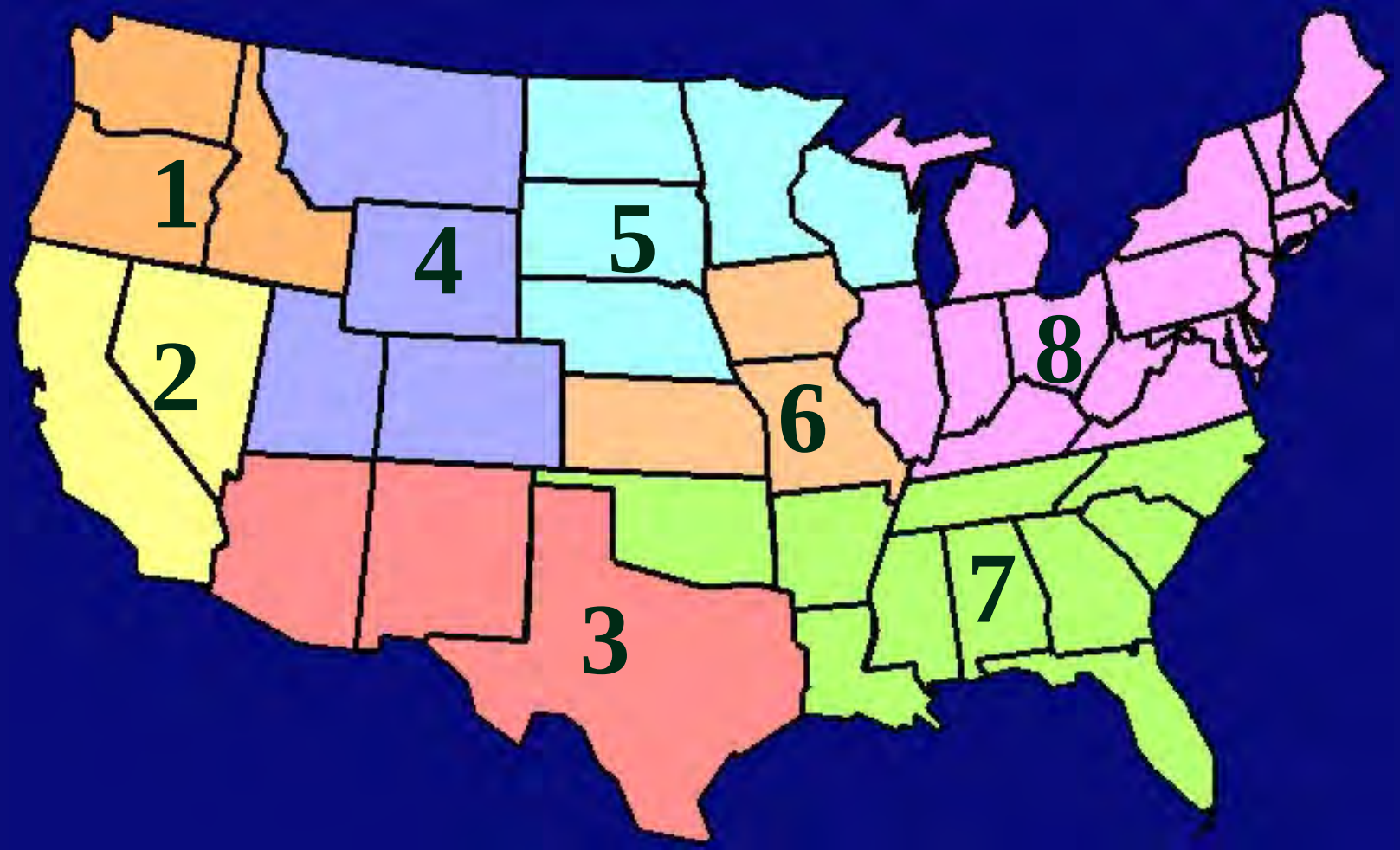
Underlined serotypes have been associated with human illness.

Bolded serotypes have been associated with Hemolytic Uremic Syndrome (HUS).

What is the Prevalence in the Ground Beef Supply?

A national survey of the prevalence of non-O157 Shiga toxin-producing *E. coli* in ground beef

BIFSCo Database Microbiological Regions



Ground beef non-O157 STEC screening and isolation results

Total samples screened	3668	of 4136 in study
positive for stx1 and/or stx2 by PCR	962	
samples with 1 or more STEC isolated	285/962	
samples with STEC isolate in top 6 non-O157 O-serogroups	13/223	

Ground beef STEC isolate molecular serotypes

Source	STEC isolates	Identified serotypes (#)							
		O26	O103	O113	O117	O121	O145	O146	other
Ground Beef	246*	**1**	**5**	36	1	**7**	0	1	196

* Only 223 of 285 isolate positive samples characterized to date

The CDC top 6 list

Virulence gene distribution of the 13 STEC isolates from Ground Beef in top 6 CDC O-Groups

STEC virulence factors	# of Isolates
*stx*1	4
*stx*2	1
*stx*1, *stx*2	0
*stx*1, *hlyA*	0
*stx*2, *hlyA*	4
*stx*1, *stx*2, *hlyA*	0
*stx*1, *eae*, *hlyA*	4
*stx*2, *eae*, *hlyA*	0
*stx*1, *stx*2, *eae*, *hlyA*	0
Total	13

Summary

- % of *stx* positive 26.2%
- % the top 6 CDC 5.8%
- % the top 6 CDC 1.8% (*stx*1)
  most likely to cause disease

Summary and Conclusions

- STEC are a natural part of the animal microflora.
- Some Non-O157 STEC can cause severe disease in humans.
- Non-O157 STEC is found at high frequency in pre-harvest samples (feces and hides).
- Non-O157 STEC is probably just as prevalent, maybe more, than O157 STEC in pre-harvest samples.
- Interventions used at the processing plants affect STECs similarly.

Summary and Conclusions

- A very small proportion of the non-O157 STECs (11.3, 7.3, 0.40, and 2.0%) have the combination of virulence factors that provide the maximum likelihood of causing disease.
- In 10,159 samples (carcass, trim and ground beef), we have detected the top 6 CDC serotypes only from 15 samples; a fraction of these have the ability to cause disease.
- To the best of our knowledge, there has never been a meat-borne non-O157 STEC outbreak in the United States.

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