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TRENDS IN TOXIN PROFILES OF HUMAN SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) O157 STRAINS, UNITED STATES, 1996-2008

BY

MOLLY M. LEEPER

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March 20th, 2009

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ABSTRACT

Molly M. Leeper
Trends in Toxin Profiles of Human Shiga Toxin-Producing *Escherchia coli* (STEC) O157 Strains, United States, 1996-2008
(Under the direction of Dr. Karen Gieseker, faculty member)

Shiga toxin-producing *E. coli* (STEC) cause diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS). All STEC produce one or both of two Shiga toxins, Stx1 and Stx2. STEC strains that produce Stx2 are more strongly associated with HUS than strains that produce Stx1 or both Stx1 and Stx2. Epidemiologic evidence indicates a recent increase in the rate of HUS among STEC outbreaks. The increasing rate of HUS could be explained by a shift in the toxin profiles of STEC strains. The purpose of this study was to examine trends in toxin profiles of human STEC O157 isolates from 1996 to 2008 and to assess whether an increase in the number of Stx2-only-producing strains could be correlated with a recent increase in HUS cases. Data from three independent datasets, collected from PulseNet, eFORS and NARMS, were used. Additionally, trends such as seasonal variations, geographical variations, gender differences, and age differences were examined for each toxin profile. Results from this study show a shift in the toxin profile of human STEC O157 strains in the United States, in that the proportion of Stx2-only producing strains has increased dramatically since 1996.

INDEX WORDS: *E. coli* O157:H7, Shiga toxin-producing *E. coli* (STEC), Hemolytic Uremic Syndrome (HUS), Shiga Toxin

TABLE OF CONTENTS

PAGE

ACKNOWLEDGEMENTS iii

LIST OF ACRONYMS AND ABBREVIATIONS iv

LIST OF TABLES v

LIST OF FIGURES vi

CHAPTER

I. **INTRODUCTION**
- Background 1
- Study Rationale 4
- Research Questions 5
- Hypotheses 6

II. **LITERATURE REVIEW**
- Public Health Significance 8
- STEC Nomenclature 11
- Human Illness 12
- Animal Illness 13
- Hemolytic Uremic Syndrome (HUS) 14
- Treatment and Prevention 16
- Detection and Isolation of STEC 18
- STEC and Adherence to Epithelial Cell Surfaces 19
- Differences in Toxin Types 22
- Toxin Subtypes 23
- STEC Serotypes 26
- Molecular Subtyping and PulseNet 28
- Electronic Foodborne Outbreak Reporting System (eFORS) 31
- National Antimicrobial Resistance Monitoring System (NARMS) 32
- STEC in the Food Chain 33

III. **METHODOLOGY**
- PulseNet Dataset 36
- eFORS Dataset 40
- NARMS Dataset 41
- Microbiological Methods 43
- Data Analysis 46

IV. **RESULTS**

PulseNet Dataset
- Distribution of Toxin Types 47
- Age Distribution 52
- Gender Distribution 53
- Seasonal Distribution 56
- Geographic Distribution 57
- Distribution of Toxin Types by PFGE 60
- Distribution of Toxin Types Among Non-O157 STEC Isolates 63

eFORS Dataset
- Number of STEC Outbreaks Within eFORS Dataset 65
- HUS Rates of Outbreaks Within eFORS Dataset 66

NARMS Dataset
- Distribution of Toxin Types Within NARMS Dataset 70
- Stx2 Toxin Subtypes Within NARMS Dataset 72

V. **DISCUSSION**
Study Significance 74
Important Study Findings 74
Study Limitations 82
Future Studies and Recommendations 83

VI. **CONCLUSIONS** 85

REFERENCES 88

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LIST OF ACRONYMS & ABBREVIATIONS

CDC	**Centers for Disease Control and Prevention**
CT-SMAC	**Cefixime and Tellurite Sorbitol-MacConkey (agar)**
eae	***E. coli* attaching and effacing gene**
eFORS	**Electronic Foodborne Outbreak Reporting System**
FBDO	**Foodborne Disease Outbreak**
FDA	**Food and Drug Administration**
GI	**Gastrointestinal**
HC	**Hemorrhagic colitis**
HUS	**Hemolytic Uremic Syndrome**
IMS	**Immunomagnetic Separation**
LEE	**Locus of enterocyte effacement**
LPS	**Lipopolysaccharide**
NARMS	**National Antimicrobial Resistance Monitoring System**
NM	**Non-motile**
NORS	**National Outbreak Reporting System**
PAI	**Pathogenicity Island**
PCR	**Polymerase Chain Reaction**
PFGE	**Pulsed-Field Gel Electrophoresis**
RFLP	**Restriction Fragment Length Polymorphism**
SMAC	**MacConkey (agar)**
STEC	**Shiga toxin-producing *E. coli***
Stx	**Shiga toxin**
TTP	**Thrombotic Thrombocytopenic Purpura**
USDA-FSIS	**United States Department of Agriculture Food Safety and Inspection Service**
VTEC	**Verocytotoxin-producing *E. coli***

LIST OF TABLES

Table Number	Title	Page Number
1	Incidence of laboratory-confirmed bacterial and parasitic infections (2007) and post-diarrheal HUS (2006)	9
2	Toxin profiles and virulence factors of most prevalent non-O157 STEC serotypes isolated from humans, United States, 1983-2002	28
3	Top 10 PFGE patterns (*Xba*I) in the *E. coli* national database	45
4	Distribution of Toxin Types, 1999-2008: [PulseNet Dataset, (n=4402)]	47
5	Distribution of Toxin Types, Sporadic Isolates, 2002-2008: [PulseNet Dataset, (n=2519)]	49
6	Distribution of Toxin Types, Outbreak Isolates, 2002-2008: [PulseNet Dataset, (n=1629)]	50
7	Age and Gender Distribution of Toxin Type Stx1-only, 1999-2008 (USA) [PulseNet Dataset, (n=51;age, n=54;gender)]	54
8	Age and Gender Distribution of Toxin Type Stx1+Stx2, 1999-2008 (USA) [PulseNet Dataset, (n=1844;age, n=1847;gender)]	55
9	Age and Gender Distribution (Median Age in Years) of Toxin Type Stx2-only, 1999-2008 (USA) [PulseNet Dataset, (n=1819; age, n=1744;gender)]	55
10	Number of Isolates with Top 6 Non-O157 STEC Serotypes (USA), 1999-2008 [PulseNet Dataset, (n=1422)]	64
11	Distribution of Toxin Types among *E. coli* O121 isolates (USA), 2003-2008 [PulseNet Dataset, (n=101)]	65
12	Number of STEC O157 Outbreaks Submitted to eFORS with Available HUS Rates, 1998-2006 [eFORS Dataset, (n=166)]	67
13	HUS Rates of STEC O157:H7 Outbreaks Submitted to eFORS with Available Toxin Information, 1998-2006 [eFORS Dataset, (n=43)]	69
14	Number of Isolates with each Toxin Type, 1996-2005 [NARMS Dataset, (n=363)]	70
15	Stx2 Toxin Subtypes of STEC O157 Isolates (na: no amplification), 1996-2005 [NARMS Dataset, (n=363)]	72

LIST OF FIGURES

Figure Number	Title	Page Number
1	Relative rates of laboratory-confirmed infections with *Campylobacter*, STEC O157, *Listeria, Salmonella,* and *Vibrio* compared with 1996-1998 rates, by year	10
2	Illustration of STEC O157 pathway of disease	13
3	Illustration of the LEE pathogenicity island (PAI) within STEC O157 strain	20
4	Adherence of STEC O157 bacteria to intestinal epithelial cell	21
5	Illustration of A an B Toxin Subunits	22
6	Dendrogram of Stx1 subtypes: 4 subtypes (Stx1a-d)	24
7	Dendrogram of Stx2 subtypes: 7 subtypes (Stx1a-g)	25
8	PFGE Gel Image of STEC O157 Isolates Restricted with *Xba*I and *Bln*I	29
9	Distribution of Toxin Types (percentage of isolates), 1999-2008: [PulseNet Dataset, (n=4402)]	47
10	Distribution of Toxin Types, Sporadic Isolates (percentage of isolates), 2002-2008: [PulseNet Dataset, (n=2519)]	49
11	Distribution of Toxin Types, Outbreak Isolates (percentage of isolates), 2002-2008: [PulseNet Dataset, (n=1629)]	50
12	Number of STEC O157 Outbreaks per year, 2002-2008 and Average Number of Cases in Outbreaks (USA), 2002-2008 [PulseNet Dataset, (n=232 outbreaks)]	51
13	Number of STEC O157 Cases Among Age Intervals (USA), 1999-2008 [PulseNet Dataset, (n=3714)]	53
14	Seasonal Distributions of Toxin Types by Quarter, 1999-2008 (USA) [PulseNet Dataset, (n=4312)]	56

15	**Overall Geographic Distribution of Isolates with Stx1-only, 1999-2008 (USA) [PulseNet Dataset, (n=67)]**	**58**
15A&B	**Geographic Distribution of Isolates with Stx1-only, 1999 and 2008 (USA) [PulseNet Dataset, (1999 n=2; 2008 n=9)]**	**58**
16	**Overall Geographic Distribution of Isolates with Stx1+Stx2, 1999-2008 (USA) [PulseNet Dataset, (n=2265)]**	**59**
16A&B	**Geographic Distribution of Isolates with Stx1+Stx2, 1999 and 2008 (USA) [PulseNet Dataset, (1999 n=74; 2008 n=389)]**	**59**
17	**Overall Geographic Distribution of Isolates with Stx2-only, 1999-2008 (USA) [PulseNet Dataset, (n=2042)]**	**60**
17A&B	**Geographic Distribution of Isolates with Stx2-only, 1999 and 2008 (USA) [PulseNet Dataset, (1999 n=10; 2008 n=532)]**	**60**
18	**Top 10 *E. coli* PFGE patterns (*Xba*I) and their Toxin Types (USA), 1999-2008, [PulseNet Dataset, (n=1676)]**	**61**
19	**Top 10 *E. coli* PFGE patterns (*Xba*I) and their Toxin Types (USA), 1999-2003, [PulseNet Dataset, (n=188)]**	**62**
20	**Top 10 *E. coli* PFGE patterns (*Xba*I) and their Toxin Types (USA), 2004-2008, [PulseNet Dataset, (n=1488)**	**63**
21	**Distribution of Toxin Types among Top 6 Non-O157 STEC Serotypes (USA), 1999-2008 [PulseNet Dataset, (n=1422)]**	**64**
22	**Number of STEC O157 Outbreaks Reported to eFORS, 1998-2006 [eFORS Dataset, (n=233 outbreaks)]**	**66**
23	**Average HUS Rates of STEC O157:H7 Outbreaks Submitted to eFORS, 1998-2006 [eFORS Dataset, (n=166)]**	**68**
24	**Distribution of Toxin Types (percentage of isolates), 1996-2005 [NARMS Dataset, (n=363)]**	**71**
25	**Stx2 Toxin Subtypes of STEC O157 Isolates (percentage of isolates) (na: no amplification), 1996-2005 [NARMS Dataset, (n=363)]**	**73**

CHAPTER I: INTRODUCTION

Background:

Escherichia coli are a group of bacteria whose members are typically non-pathogenic normal microflora of the intestinal tract of humans and animals. However, certain strains of this bacterial species have acquired genes that enable them to cause intestinal disease. The *E. coli* that cause enteric disease have been divided into pathotypes based on their virulence factors and mechanisms by which they cause disease. One of these pathogens, called Shiga toxin-producing *E. coli*, refers to those strains of *E. coli* that produce at least one member of a class of potent cytotoxins called Shiga toxins (Gyles 2006).

During the past two decades, an increasing number of human foodborne illness outbreaks have been traced to consumption of undercooked ground beef and other beef products contaminated with Shiga toxin-producing *Escherichia coli* O157 (STEC). STEC, also referred to as Verocytotoxin-producing *E. coli* (VTEC) are causes of major, potentially fatal, zoonotic food-borne illness whose clinical spectrum includes diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Karmali 2003). STEC infections are considered a public health problem in both developed and developing countries because of the severity of the disease they cause and the global nature of the food supply (Brando 2008)**.**

Shiga toxin-producing *Escherichia coli* (STEC) is a major cause of foodborne illness in the United States, and is usually acquired by ingestion of contaminated food or water, contact with animals, or by person-to-person transmission. Sources of STEC

infection in humans include foods of animal origin such as meats (especially ground beef), unpasteurized milk, and other vehicles that have been contaminated with STEC, such as fresh-pressed apple cider, yogurt, and vegetables such as alfalfa sprouts, lettuce, and other leafy greens. Waterborne transmission and contact with infected animals are two routes of transmission that are becoming increasingly recognized. In addition to large, widespread outbreaks in the United States, outbreaks of STEC infection have been documented in at least 14 countries in a variety of settings, including households, daycare centers, schools, restaurants, nursing homes, and prisons (Karmali 2003).

STEC causes severe gastroenteritis and may cause life-threatening HUS, the most serious complication of STEC infection. Most patients with HUS in developed countries have evidence of exposure to Shiga toxin-producing *E. coli* (O'Brien 1998). HUS is a leading cause of acute renal failure in children and occurs in about 6% of patients with STEC infection (Griffin 1998). Up to 40% of patients with HUS develop long-term renal dysfunction and about 3-5% of patients die during the acute phase of the disease (Karmali 2003).

Although the main virulence factor of STEC is the production of one or more type of *Shiga* toxin (Stx1, Stx2, or both), adherence to the intestinal epithelium and colonization of the gut are also important components of the disease. Although STEC is not typically invasive and is restricted to the lumen of the gut, in some circumstances Shiga toxin (Stx) produced within the intestinal tract is able to cross the epithelial border and enter the bloodstream. Both Stx1 and Stx2 are capable of crossing epithelial borders via an energy-requiring process, and the toxin that moves across the border retains its biological activity. Stx targets the endothelium of susceptible tissues, resulting in

intestinal as well as systemic dysfunction (Brando 2008). While the route that the toxin uses to pass across epithelial cell barriers is not well understood, it appears to take a transcellular route. This notion is based on the observation that toxin movement is energy dependent and directional, with greater toxin movement in the apical-to-basolateral direction than vice versa (Acheson 1998).

Molecular subtyping, such as pulsed-field gel electrophoresis (PFGE), is critical in linking widely dispersed outbreaks of STEC. Subtyping can link seemingly sporadic cases so that a vehicle can be implicated and public health officials and consumers can advocate for changes to make food safer. Molecular subtyping has had several major impacts on public health, including increasing the ability to identify outbreaks that would otherwise be missed, increasing the specificity of the definition of outbreaks, and allowing outbreaks to be detected and controlled at an earlier stage (Tauxe 2006).

To facilitate epidemiologic investigations, the Centers for Disease Control and Prevention (CDC) established a national molecular suptyping network for foodborne disease surveillance in the United States, known as PulseNet. Since its inception in 1996, PulseNet has been instrumental in the detection, investigation, and control of numerous outbreaks caused by STEC and other foodborne disease-causing bacteria. A server housed at the CDC holds a national database of STEC isolates submitted by state and local health departments in the United States. By rapidly detecting clusters of STEC infections, the *E. coli* national database is a key tool in the recognition and investigation of outbreaks (Gerner-Smidt 2006).

Additionally, since 1973, CDC has maintained a collaborative surveillance program for collection and periodic reporting of data on the occurrence and causes of

foodborne-disease outbreaks (FBDOs) in the United States. The Foodborne Disease Outbreak Surveillance System reviews data on FDBOs, defined as the occurrence of two or more cases of a similar illness resulting from the ingestion of a common food. State and local public health departments have the primary responsibility for identifying and investigation FDBOs. These departments use a standard form to report these outbreaks. Since 2001, reports of FDBOs are submitted through a web application on the internet called the Electronic Foodborne Outbreak Reporting System (eFORS) (Lynch 2006). In 2007 the eFORS system began undergoing developmental changes, and will soon be replaced by the National Outbreak Reporting System (NORS). While eFORS collects outbreak data on foodborne outbreaks, NORS will integrate foodborne, waterborne, zoonotic, and person-to-person enteric disease outbreaks. It is estimated that NORS will be deployed in early 2009 (Ayers 2008).

Study Rationale:

The motivation for this study comes from an observed increase in the number of *E. coli* O157:H7 outbreaks in the United States population and the increased rate of HUS within these outbreaks. Epidemiologic evidence in Europe shows a recent increase in the rate of HUS among cases involved in STEC outbreaks (Werber 2003). The increasing rate of HUS could be explained by a shift in the toxin profiles (Stx1, Stx2, and Stx1+2) of STEC strains.

The purpose of this study is to examine trends in toxin profiles (Stx1, Stx2, or both) of human STEC O157 isolates from 1999 to 2008 and to assess whether an increase

in the number of Stx2-producing strains is correlated with the recent increase in HUS cases in outbreaks.

Research Questions:

From initial observations and review of the literature, the following research questions were formulated:

1) Has there been an increase in the number of STEC O157:H7 outbreaks within the time period observed (1999-2008)? If so, what factors could be affecting these numbers?

2) Has there been an increase in the number of STEC O157:H7 strains that produce Shiga toxin 2-only within the time period observed (1999-2008)? If such an increase exists, have there been any changes in toxin testing practices?

3) Has there been an increase in the rate of HUS among STEC outbreaks occurring in the United States from the time period observed (1999-2008)?

4) Are there any other observable trends in the number of STEC O157:H7 outbreaks and HUS rates within the time period observed (1999-2008), including demographic, seasonal, or geographic trends?

5) Could trends in HUS rates be due to shifts in the toxin profiles produced by STEC O157:H7 strains?

6) Are certain PFGE patterns associated with certain toxin profiles in STEC O157:H7 strains?

7) Are certain toxin profiles associated with non-O157 STEC strains?

Hypotheses:

The following hypotheses were generated from the questions proposed, general observations, the literature reviewed, and the data collected:

1) H_O: There has not been a change in the number of foodborne outbreaks caused by STEC O157:H7 reported during the time period of 1999-2008.

   H_A: There has been an increase in the number of foodborne outbreaks caused by STEC O157:H7 reported from the time period of 1999-2008.

2) H_O: There has not been a relative increase in the number of STEC O157:H7 strains that produce Shiga toxin 2-only from the time period of 1999-2008.

   H_A: There has been a relative increase in the number of STEC O157:H7 strains that produce Shiga toxin 2-only from the time period of 1999-2008.

3) H_O: There has not been an increase in the rate of HUS among STEC O157 outbreaks reported in the United States from the time period observed (1999-2008).

   H_A: There has been an increase in the rate of HUS among STEC O157 outbreaks in the United States from the time period observed (1999-2008).

4) H_O: There are no other observable trends in the number of STEC O157:H7 outbreaks and HUS rates within the time period observed (1999-2008), including demographic, seasonal, or geographic trends.

   H_A: There are other observable trends in the number of STEC O157:H7 outbreaks and HUS rates within the time period observed (1999-2008), including demographic, seasonal, or geographic trends. Trends in age and gender include higher rates of STEC O157:H7 infections among young children (<5 years of age)

and those of female gender. STEC O157:H7. Seasonal trends include higher rates of STEC O157:H7 infection in summer and fall months, for each toxin type. Geographic trends include higher concentrations of STEC O157:H7 cases in mid-western and mountain states.

5) H_O: PFGE patterns do not correlate with the toxin profile of STEC O157:H7 strains.

   H_A: PFGE patterns correlate with the toxin profile of STEC O157:H7 strains.

6) H_O: Non-O157 STEC strains are not more likely to be associated with the toxin profile Stx1-only than the other two toxin profiles.

   H_A: Non-O157 STEC strains are more likely to be associated with the toxin profile Stx1-only than the other two toxin profiles

CHAPTER II: LITERATURE REVIEW

Public Health Significance of STEC O157:H7 Infections

The occurrence of massive outbreaks of STEC infection, especially resulting from the most common serotype, *E. coli* O157:H7, and the risk of developing HUS, the leading cause of acute renal failure in children, make STEC infection a public health problem of serious concern (Karmali 2003). Since the first outbreak caused by *E. coli* O157:H7 in 1982, this agent has emerged as a foodborne pathogen leading to hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombocytopenic purpura (TTP) (O'Brien 1998). The main virulence factor of STEC is the production of one or more type of Shiga toxin, (Stx1, Stx2, or both).

Recent epidemiologic evidence indicates that the incidence of infections with STEC O157:H7 and other strains has increased in the 1980s and 1990s. In 1999, it was estimated that STEC O157:H7 causes approximately 73,000 illnesses and 61 deaths annually in the United States (Mead, 1999). The Foodborne Diseases Active Surveillance Network (FoodNet) of CDC's Emerging Infections Program collects data from 10 states regarding diseases caused by pathogens commonly transmitted through food. FoodNet quantifies and monitors the incidence of these infections by conducting active, population-based surveillance for laboratory-confirmed infections. In 2007, 545 cases of STEC O157 were identified in FoodNet surveillance areas, yielding an incidence of 1.20 cases per a population of 100,000 with large geographical variation. The highest incidence for STEC O157 infections was among children aged <5 years (3.66 cases per a

population of 100,000). In 2006, FoodNet identified 82 cases of post-diarrheal HUS in persons aged <18 years (0.78 cases per 100,000 children); 58 (0.7%) cases occurred in children aged <5 years (2.01 cases per 100,000 children). Table 1 shows the 2007 incidence of laboratory-confirmed STEC O157 infections and post-diarrheal HUS by FoodNet site, as compared to the Healthy People 2010 Objective for food safety (CDC 2008).

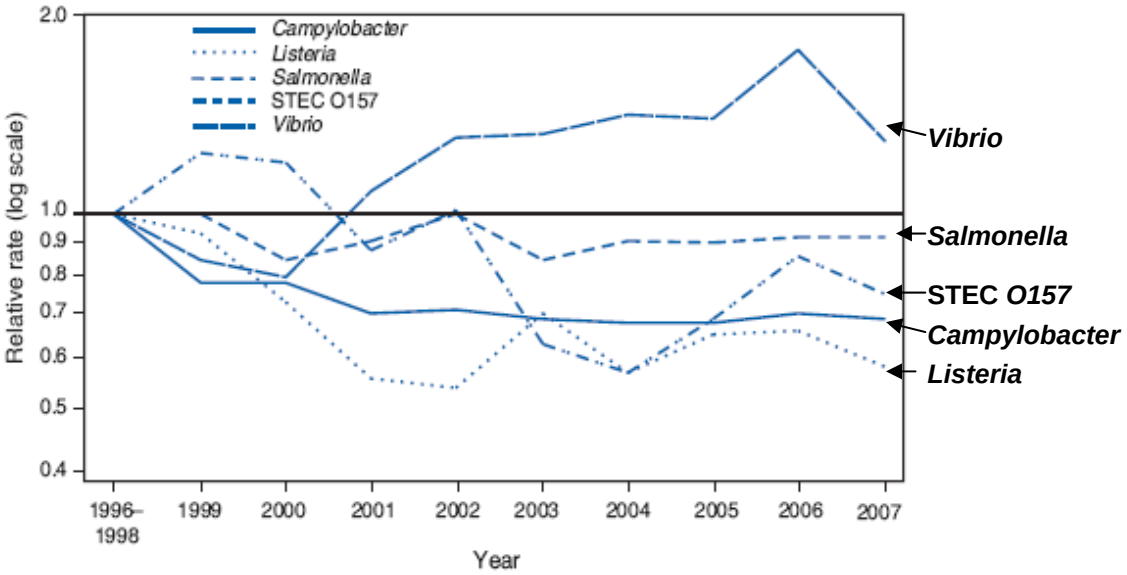
Table 1: Incidence* of laboratory-confirmed bacterial and parasitic infections in 2007 and post-diarrheal hemolytic uremic syndrome (HUS) in 2006, by site and pathogen, compared with national health objectives†. Source: MMWR 2008.

Pathogen	California	Colorado	Connecticut	Georgia	Maryland	Minnesota	New Mexico	New York	Oregon	Tennessee	Overall	National health objective
Bacteria												
Campylobacter	28.21	15.85	14.01	7.29	7.19	17.51	17.55	11.98	19.02	7.39	**12.79**	12.30
Listeria	0.25	0.34	0.34	0.33	0.27	0.14	0.20	0.26	0.24	0.26	**0.27**	0.24
Salmonella	14.29	11.99	12.27	21.78	15.33	13.74	14.38	12.09	8.65	14.13	**14.92**	6.80
Shigella	5.55	3.00	1.26	17.39	1.91	4.61	5.42	0.89	1.78	6.01	**6.26**	N/A§
STEC¶ O157	1.21	1.21	1.28	0.50	0.39	3.19	0.46	1.35	1.97	0.91	**1.20**	1.00
STEC non-O157	0.22	2.12	0.74	0.44	0.46	0.74	1.28	0.28	0.14	0.40	**0.57**	N/A
Vibrio	0.37	0.15	0.46	0.25	0.45	0.15	0.00	0.21	0.22	0.05	**0.24**	N/A
Yersinia	0.47	0.15	0.51	0.46	0.14	0.45	0.20	0.37	0.51	0.22	**0.36**	N/A
Parasites												
Cryptosporidium	1.24	3.87	1.20	2.45	0.57	5.81	6.14	2.07	3.51	2.19	**2.67**	N/A
Cyclospora	0.03	0.00	0.09	0.03	0.02	0.00	0.10	0.05	0.00	0.02	**0.03**	N/A
HUS**	2.36	2.50	1.48	1.00	0.81	2.32	—	0.43	2.60	5.02	**2.01**	0.90
Surveillance population (millions)	3.23	2.64	3.50	9.36	5.62	5.17	1.95	4.29	3.70	6.04	**45.50**	

* Per 100,000 population.
† Healthy People 2010 objective 10 targets for incidence of *Campylobacter*, *Salmonella*, and Shiga toxin-producing *Escherichia coli* O157 infections and HUS for 2010 and for incidence of *Listeria* infections for 2005 and 2010, as revised by midcourse review.
§ No national health objective exists for these pathogens.
¶ Shiga toxin-producing *Escherichia coli*.
** Incidence of postdiarrheal HUS in children aged <5 years; denominator is surveillance population aged <5 years in sites that conduct hospital discharge data review.

The relative rates of laboratory-confirmed infections of STEC O157 and other foodborne pathogens (*Campylobacter, Listeria, Salmonella, and Vibrio*) from 1996-2007 according to data collected by FoodNet is shown in Figure 1.

Figure 1: Relative rates of laboratory-confirmed infections with *Campylobacter*, STEC* O157, *Listeria, Salmonella,* and *Vibrio* compared with 1996-1998 rates, by year. Foodborne Diseases Active Surveillance Network, United States, 1996-2007†. Source: MMWR 2008.



* Shiga toxin-producing *Escherichia coli*.
† The position of each line indicates the relative change in the incidence of that pathogen compared with 1996–1998. The actual incidences of these infections can differ.

Although significant declines in the incidence of certain foodborne pathogens have occurred since 1996, these declines mainly occurred before 2004. Declines in the incidence of STEC O157 infections in 2003 and 2004 have not been maintained.

Although the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) and the beef processing industry have implemented interventions to reduce ground beef contamination, 21 beef product recalls for possible contamination with STEC O157 were issued in 2007, of which 10 were illness associated, an increase compared to previous years. USDA-FSIS launched an STEC O157 initiative in fall 2007 and hosted a public meeting in spring 2008 to explore solutions to the challenges the pathogen presents (CDC 2008).

Shiga Toxin Nomenclature and Verotoxins

In 1898, Kiosha Shiga described the agent of epidemic bacterial dysentery, *Shigella dysenteriae* type 1 (Shiga's bacillus). Shiga's bacillus was later found to produce Shiga toxins. In 1972, Keusch and colleagues found that Stx alone caused fluid accumulation and enteritis in rabbit intestines, revealing that Stx can contribute to bloody diarrhea. In 1977, Konowalchuck and colleagues made the critical finding that certain diarrheagenic *E. coli* stains make a cytotoxin that can kill Vero cells (cells derived from the kidney epithelial cells of the African green monkey), hence the name verotoxin. In 1983, O'Brien and colleagues reported that a Shiga-like toxin was produced by the *E. coli* O157:H7 strain that had caused an outbreak of hemorrhagic colitis in the United States, and that this toxin was the same as the verotoxin produced by *E. coli* O157:H7. Thus, in 1983, the paths of research on Shiga toxins and verotoxins merged. Following these significant findings, the mid to late 1980s heralded the era of the molecular characterization of the genes encoding the Stx family members and it was shown that the Shiga toxin from *Shigella dysenteriae* belonged to the Shiga toxin type (O'Brien 1998).

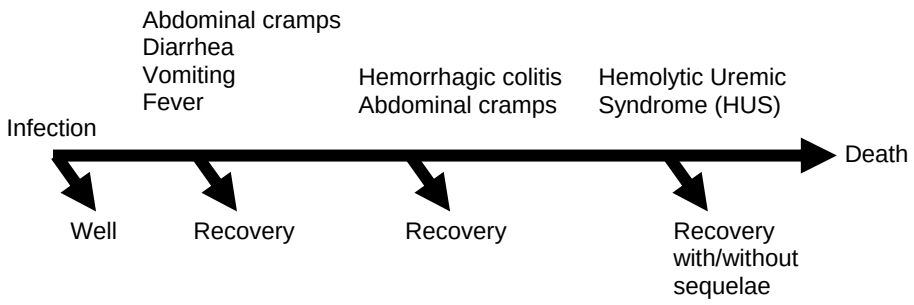
Two main categories of Shiga toxins have been distinguished, *E. coli* Shiga toxin 1 (Stx1) is almost identical to the Shiga toxin of *Shigella dysenteriae* in amino acid sequence, whereas Shiga toxin 2 (Stx2) is less related to the Shiga toxin of *Shigella* and is not neutralized by antibodies to either Stx1 or Shiga toxin from *S. dysenteriae* (Boerlin 1998).

Human Illness

In outbreaks of STEC O157:H7, the mode of transmission is most often food, followed by animal contact, person-to-person spread, recreational water, and drinking water. STEC infection typically occurs in the summer and fall and mostly affects young children, but the elderly also have an increased risk of infection. The infectious dose is very low, estimated to be less than 100 to a few hundred organisms (Griffin 1998). The sequence of events of STEC O157:H7 infection begins with the ingestion of the organism, followed typically by a 3-4 day incubation period while it colonizes the large bowel and multiplies. Illness then begins with non-bloody diarrhea and abdominal cramps. Most persons who come to medical attention develop bloody diarrhea, typically in the 2nd or 3rd day of illness. Illness usually resolves within a week, but in about 6% of patients HUS occurs. Fever and vomiting are not prominent features (Griffin 1998). Approximately 10% of patients with STEC O157:H7 infections do not experience bloody diarrhea, however, patients with non-bloody diarrhea have the same risk of developing HUS as do patients with hemorrhagic colitis, and they are as severely affected in terms of abdominal pain and other symptoms (Tarr 1998). Figure 2 illustrates the range in

symptomatology of STEC disease from asymptomatic infection to death, and the potential symptoms along its progression**.**

Figure 2: STEC O157 pathway of disease. Source: STEC – Role of Clinical and Public Health Microbiologists in Testing and Outbreak Situations. Source: STEC – Role of Clinical and Public Health Microbiologists in Testing and Outbreaks. Presented by Dr. Peter Gerner-Smidt, 108th ASM General Meeting, Boston 2008



Animal Illness

Healthy dairy and beef cattle are the major reservoir of a diverse group of STEC that infects humans through contamination of food and water, as well as through direct contact (Gyles 2006). Naturally acquired STEC infections have also been detected in a wide spectrum of animal species (sheep, goat, deer, moose, swine, horse, dog, cat, pigeon, chicken, turkey). Several of these animal hosts, particularly ruminants, have been identified as major reservoirs of STEC strains that are highly virulent in the human host, including STEC O157:H7 (Wieler 2003) .

However, in contrast to the human host, most STEC infections of animals remain clinically inapparent. Even in ruminant species, where high shedding rates have been reported, the clinical significance of STEC infections appears to be rather limited. Calves

are infected soon after birth through fecally contaminated milk and surroundings. Studies show that STEC O157 strains are only pathogenic for animals younger than 3 weeks, a finding pointing toward a possible age-dependent expression of STEC-specific intestinal receptors in animals. Similar to the diagnostic approach in human STEC infection, a definitive diagnosis in animals is based on the isolation of the bacteria from fecal specimens and subsequent confirmation by the demonstration of virulence factors or their genes (Wieler 2003).

Hemolytic Uremic Syndrome (HUS)

HUS, which was first described in 1955 by Gasser et al. in Switzerland, is defined by a triad of clinical features that include renal failure, thrombocytopenia, and microangiopathic hemolytic anemia. Before 1983, most nephrologists thought HUS was a multifactorial disease that could result from a number of initiating events. Because HUS occasionally occurred in outbreaks, an infectious agent was sought. The strongest documented linkage between HUS and a microorganism was the association of *Shigella dysenteriae* type 1. Several studies had noted that many, if not the majority, of HUS cases were preceded by diarrhea. The key event in the linkage of HUS and STEC was the report by Karmali et al. in 1983 that sporadic cases of HUS were linked to the presence of Stx and/or *E. coli* that produced Stx in patients' stools. This initial report was confirmed by a prospective controlled study that linked cases of HUS with isolation from the stools of patients with STEC infections belonging to at least 6 different O serogroups (O26, O111, O113, O121, O145, and O157). Subsequent reports also noted an association between STEC and post-diarrheal TTP, a syndrome more commonly found in adults that

shares many features of HUS (O'Brien 1998). The development of HUS is thought to be related to the translocation of Stx into the bloodstream, although the precise mechanism for this is unknown (Karmali 2003).

The severity of HUS varies from an incomplete or mild clinical picture to severe and fulminating disease with multiple organ involvement, including the bowel, heart, lungs, pancreas, and central nervous system (Karmali 2003). Neurological complications such as seizures, stroke, cerebral edema, or coma may occur in HUS, but there is little information on the pathophysiology of the central nervous system (O'Brien 1998). Approximately two-thirds of children with HUS require dialysis, and about one-third have milder renal involvement without the need for dialysis. The use of anti-motility agents and antibiotics, having bloody diarrhea, fever, vomiting, elevated serum leukocyte count, being of a young age (<5 years) and of female gender have been associated with increased risk for HUS following STEC infection in some studies (Scheiring 2008).

HUS has been reported to occur with a frequency of about 8% in several outbreaks of *E. coli* O157:H7, although in one outbreak among elderly nursing home residents, it was as high as 22%. Recently, a large, well-publicized multistate outbreak associated with fresh spinach consumption occurred in September 2006 across 26 states with approximately 200 illnesses and 3 deaths. The HUS rate for this outbreak was found to be 15.6% (CDC 2006).

The incidence of HUS in North America is about three cases per 100,000 children under 5 years of age per year; the rate among older children is somewhat lower, and the rate among adults is not known (Mahon 1997). This is in contrast to a roughly 10-fold higher incidence (consistently) in children under 5 years of age in Argentina. In South

Africa and the US, HUS appears to be more common in white than in black children. In Argentina, HUS occurs more commonly in upper-income than in lower-income groups. Reasons for these differences are unknown (Karmali 2003).

Treatment and Prevention

In an outbreak setting, rapid diagnosis of cases and immediate notification of health authorities is essential for effective intervention. The presentation to medical care of a child with definite or possible *E. coli* O157:H7 infection but before HUS ensues affords a potential opportunity to ameliorate the course of subsequent renal failure. HUS can be categorized as either oligoanuric (which probably signifies acute tubular necrosis) or nonoligoanuric. Children with oligoanuric renal failure during HUS generally require dialysis, have more complicated courses, and are probably at increased risk for chronic sequelae than are children who experience nonoligoanuric HUS (Ake 2005). A prospective study on 29 children with HUS that was confirmed microbiologically to be caused by *E. coli* O157:H7 was performed by Ake et al. This study found that early recognition and parenteral volume expansion during *E. coli* O157:H7 infections, well before HUS develops, is associated with attenuated renal failure. Parenteral hydration in children who are possibly infected with *E. coli* O157:H7, at the time of presentation with bloody diarrhea and in advance of culture results, is a practice that can accelerate the start of volume expansion during the important pre-HUS interval. Rapid assessment of stools for *E. coli* O157:H7 by microbiologists and reporting of presumptive positives immediately can alert practitioners that patients are at risk for developing HUS and can prompt volume expansion in children (Ake 2005).

The use of antibiotics to treat patients with STEC infection has been quite controversial. Most clinicians experienced in the management of STEC infections in the US and Canada have found that antimicrobial agents have little clinical effect and occasionally seem to increase the chances of HUS, however this finding is still in debate. A less controversial treatment that is being followed is the use of Synsorb-Pk, which is an investigational new drug that has been promoted as safe and effective for the treatment of HUS in children infected by *E. coli*. This drug is intended to absorb the toxin in the intestine before it reaches the bloodstream. There have been improvements in the treatment of renal failure, however the biggest challenge facing clinicians is to develop interventions to prevent renal involvement (O'Brien 1998).

Another area of investigation is the development of vaccines against STEC. Successful vaccination of pigs against edema disease, using Stx2e toxoids**,** (bacterial toxins whose toxicity has been weakened or suppressed by chemical or heat treatments), offers hope for human vaccines (O'Brien 1998). Anti-Shiga toxin antibodies have been shown to prevent HUS in animals. In December 2005, the US Food and Drug Administration (FDA) approved orphan drug status for two chimeric anti-Shiga toxin antibodies (caStx1 and caStx2, made by Caprion Pharmaceuticals, Inc.) in the treatment of STEC infections. The antibodies are intended to neutralize circulating Stx1 and Stx2, thereby preventing serious complications such as bloody diarrhea, destruction of red blood cells and platelets, and HUS. The product is being evaluated for preventing HUS in a dose-escalating, phase 1, US clinical trial of STEC infected pediatric patients (Scheiring 2008).

Detection and Isolation of STEC

Timely and accurate diagnosis of STEC infections is extremely important from both a public health and a clinical management perspective. Several days may occur between the point at which the patient is exposed to the pathogen and when he/she is included as a case in an outbreak. These include the time it takes for the patient to become ill after ingesting the contaminated food (3-4 days), the time it takes for the patient to contact the healthcare system (1-5 days), the time it takes to diagnose the infection after a stool sample is collected (1-3 days), the time it takes for the patient's specimen to be shipped from the clinical laboratory to the public health laboratory (1-7 days), and the time it takes for the public health laboratory to perform molecular testing on the patient's specimen to confirm the case as part of an outbreak (2-10 days). In cases of HUS, the typical clinical signs usually become apparent within two weeks after the onset of gastrointestinal (GI) symptoms, by which time the numbers of the causative STEC may be very low, or diarrhea may no longer be present.

For these reasons, STEC detection methods need to be very sensitive. Diagnostic methods are based on the detection of the presence of either Stx genes in fecal extracts or fecal cultures and/or isolation of the STEC itself. These procedures differ in complexity, speed, sensitivity, specificity, and cost, therefore diagnostic strategies must be tailored to the clinical circumstances and resources available (Paton J. 2003). Culture on Sorbitol-MacConkey agar (SMAC) or the more selective cefixime and tellurite sorbitol-MacConkey (CT-SMAC) agar has been the most commonly used method for isolation of STEC O157. This is because unlike the majority of fecal *E. coli* strains, most O157:H7 and O157:H- STEC are unable to ferment sorbitol. SMAC or CT-SMAC plates are

inoculated with the fecal specimen and examined after 18-24 hours of incubation for the presence of colorless, sorbitol-negative colonies. Individual colonies can then be tested by slide or tube agglutination with commercially available O157-and H7-specific antisera or latex reagents (Paton A. 2003). Although screening fecal cultures on SMAC or CT-SMAC is inexpensive and involves minimal labor and equipment, it is serotype-specific in that it will only detect STEC O157.

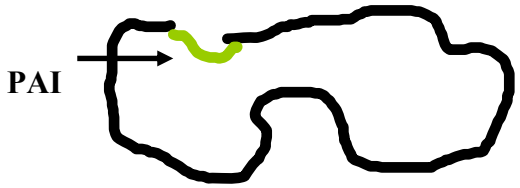
Immunomagnetic separation (IMS) is a powerful concentration technique for the isolation of STEC from low-abundance specimens. This procedure involves coating magnetic beads with anti-LPS (lipopolysaccharide) and mixing them with broth cultures or suspensions of feces or food samples. The beads and bound bacteria are then trapped in a magnetic field, any unbound suspension is decanted, and the beads are washed. After additional binding and washing cycles, the beads are plated and the resultant colonies are tested for Shiga toxin production. IMS is an extremely valuable technique in circumstances where deliberate targeting of STEC O157 is justifiable, such as for analysis of food samples that have been epidemiologically linked to human cases of STEC, and for analysis of stool cultures from patients with HUS (Paton A. 2003). IMS is also valuable for detection of the most common non-O157 STEC serotypes for which antibodies are available, e.g. O111, O26, O103, and O45.

Shiga Toxins and Adherence to Epithelial Cell Surfaces

Once STEC has been ingested, they are able to survive the acidity of the stomach in sufficient numbers to colonize portions of the lower GI tract. Once the organisms are in the lower portion of the intestine, the bacteria adhere to, and interact

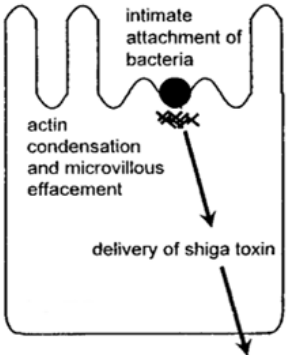
with, the epithelial cell surface by using a variety of virulence factors, some of which are encoded by genes present on a pathogenicity island (PAI) known as the locus of enterocyte effacement (LEE). A PAI is a stretch of foreign DNA that is incorporated into the genome of pathogenic microorganisms and carries genes encoding one or more virulence factors, including toxins (Figure 3). The GC content of a PAI differs from that of the rest of the genome, indicating that at some point in history the pathogen has acquired the DNA located on the PAI from an outside source (Hacker 2000).

Figure 3: Illustration of the LEE pathogenicity island (PAI) within STEC O157 strain. The PAI contains genes which encode various virulence factors for the organism. Source: STEC – Role of Clinical and Public Health Microbiologists in Testing and Outbreaks. Presented by Dr. Peter Gerner-Smidt, 108th ASM General Meeting, Boston 2008



Nearly all O157 strains contain the *E. coli* attaching and effacing (eae) gene, which mediates the attachment to and destruction of the microvilli of the intestinal epithelial cells (Bulte 2003). Once the organism adheres to the epithelial cell surface, it then produces Shiga toxins, which are capable of causing damage both locally and systemically (Acheson 1998). Figure 4 illustrates this component of STEC pathogenesis**.**

Figure 4: Adherence of STEC O157 bacteria to intestinal epithelial cell and delivery of Shiga toxin inside the cell. Source: Nataro and Kaper, 1998. Clinical Microbiological Review 11: 142-201.

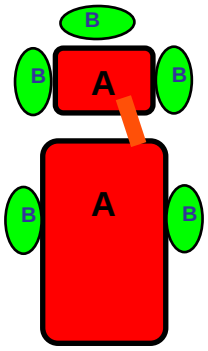


It is well established that certain patients develop endothelial cell damage in sites that are at a distance from the GI tract following infection. This is thought to be due, to some degree, to the direct action of the toxins (Acheson 1998). Shiga toxin is toxic to cells at picomolar concentrations, and they are among the most potent biological substances known (Karmali 2003).

The toxins share a polypeptide subunit structure consisting of an enzymatically active A-subunit that is linked to a pentamer of B-subunits. The A-B subunit structure binds to a specific receptor on the surface of eukaryotic cells (Nataro and Kaper 1998). Figure 5 illustrates the structure of the toxin. After binding to a receptor on the eukaryotic cell, the toxins are internalized by endocytosis. Once inside the cell, the A subunit is split and becomes activated, and the toxins target the endoplasmic reticulum by a process called “retrograde transport”, where they interact with subcellular components, resulting in the inhibition of protein synthesis and cell death. Although the endothelial cell appears to be the main target for Stx action, there is evidence that the toxins may also mediate biological effects by interacting with other cell types such as renal tubular cells and

monocytes (Karmali 2003). Endothelial cell damage is central to the pathogenesis of HUS, and damage is normally caused in the renal cells, but may also occur in the gastro-intestinal tract, as well as other organs including the pancreas, lungs, and brain.

Figure 5: The toxin has two subunits, designated A and B. The B subunit is a pentamer that binds to specific glycolipids on the host cell, specifically globotriaosylceramide (Gb3). Source: STEC – Role of Clinical and Public Health Microbiologists in Testing and Outbreaks. Presented by Dr. Peter Gerner-Smidt, 108th ASM General Meeting, Boston 2008



Differences in Toxin Types

Two types of Shiga toxins exist: Stx1 and Stx2. Some studies have suggested that STEC strains producing Stx2 may be more closely associated with severe disease and HUS than strains producing Stx1-only. In a study from the United States, patients infected with STEC O157 possessing Stx2 but not Stx1 were significantly more likely to develop systemic sequelae, including HUS, than were patients infected with STEC O157 harboring Stx1 alone or Stx1 and Stx2 (Ostroff 1989).

In 2004, Ethelberg et al. conducted an analysis of strain and patient factors associated with the development of bloody diarrhea and HUS among STEC patients registered in Denmark in a 6-year period. This study found that a major risk factor for bloody diarrhea and HUS was the presence of the Stx2 and eae genes (Ethelberg 2004). A study conducted in 1999 by Boerlin et al. revealed a strong statistical association (OR=4.95; p=0.0038) between the Stx2 gene and severity of disease for a set of 112 human isolates from eight major serotypes (Boerlin 1999).

One possible explanation for this is that Stx2 moves across the intestinal epithelial cell barrier to a greater extent that does Stx1. One study found that Stx1 binds with higher affinity than Stx2 in a number of epithelial and endothelial cells. One speculation is that if Stx1 is binding to many of the available receptors with higher affinity, it may be more likely to be “held up” in the intestine, preventing it from entering the bloodstream. It is not clear if Stx1 and Stx2 are moving across the intestinal epithelial cells via the same pathway, although it is highly probably that they are (Acheson 1998). Each Stx type may be present alone or in combination in STEC. The pathogenicity of STEC infection in humans depends on many bacterial virulence factors including among others, Stx, enterohemolysin, intimin (encoded by the eae gene), and host factors such as age.

Toxin Subtypes

Each Stx type (Stx1 and Stx2) may be further divided into several subtypes. For example, Stx1 may be divided into 4 subtypes, Stx1a, Stx1b, Stx1c, and Stx1d, and Stx2 may be divided into 7 subtypes, Stx2a-g. Stx2e typically is associated with pig edema disease and has been rarely detected in STEC of human origin (Friedrich 2002). The

Stx2a subtype is also called the Stx2 subtype by some investigators, in order to avoid confusion with the A subunit of the toxin.

The Stx subtype may be associated with the clinical presentation and severity of illness among STEC infections. A number of studies have documented that types Stx2a and Stx2c are more often associated with HUS than the other Stx2 subtypes, but Stx2d and Stx2e-containing strains have also been isolated from humans with HUS. These data suggest that some Stx2 subtypes augment the ability of STEC to cause serious human disease (Friedrich 2002). Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) has been the preferred tool for subtyping Stx2 genes. This method is, however, vulnerable to single-nucleotide changes and is difficult to interpret if the strain contains more than one subtype or if the fragments generated are small or of similar sizes (Persson 2007). Figures 6 and 7 illustrate the similarity of the different subtypes of each toxin type, Stx1 and Stx2, and how certain STEC serotypes group among the subtypes of each type. These dendrograms are amino acid sequences translated from the partial sequences of the Stx1 and Stx2 genes.

Figure 6: Stx1: 4 subtypes (Stx1a-d); 7 variants. Source: F. Scheutz, USDA, FDA, CDC: Public non-O157 meeting, Washington DC 2007.

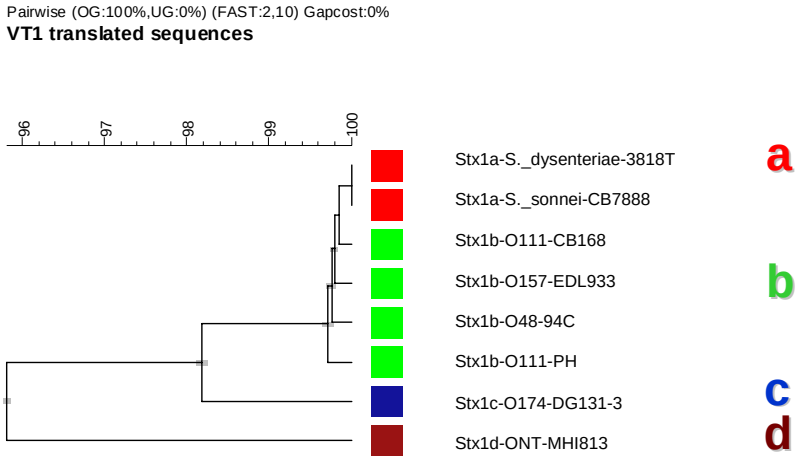
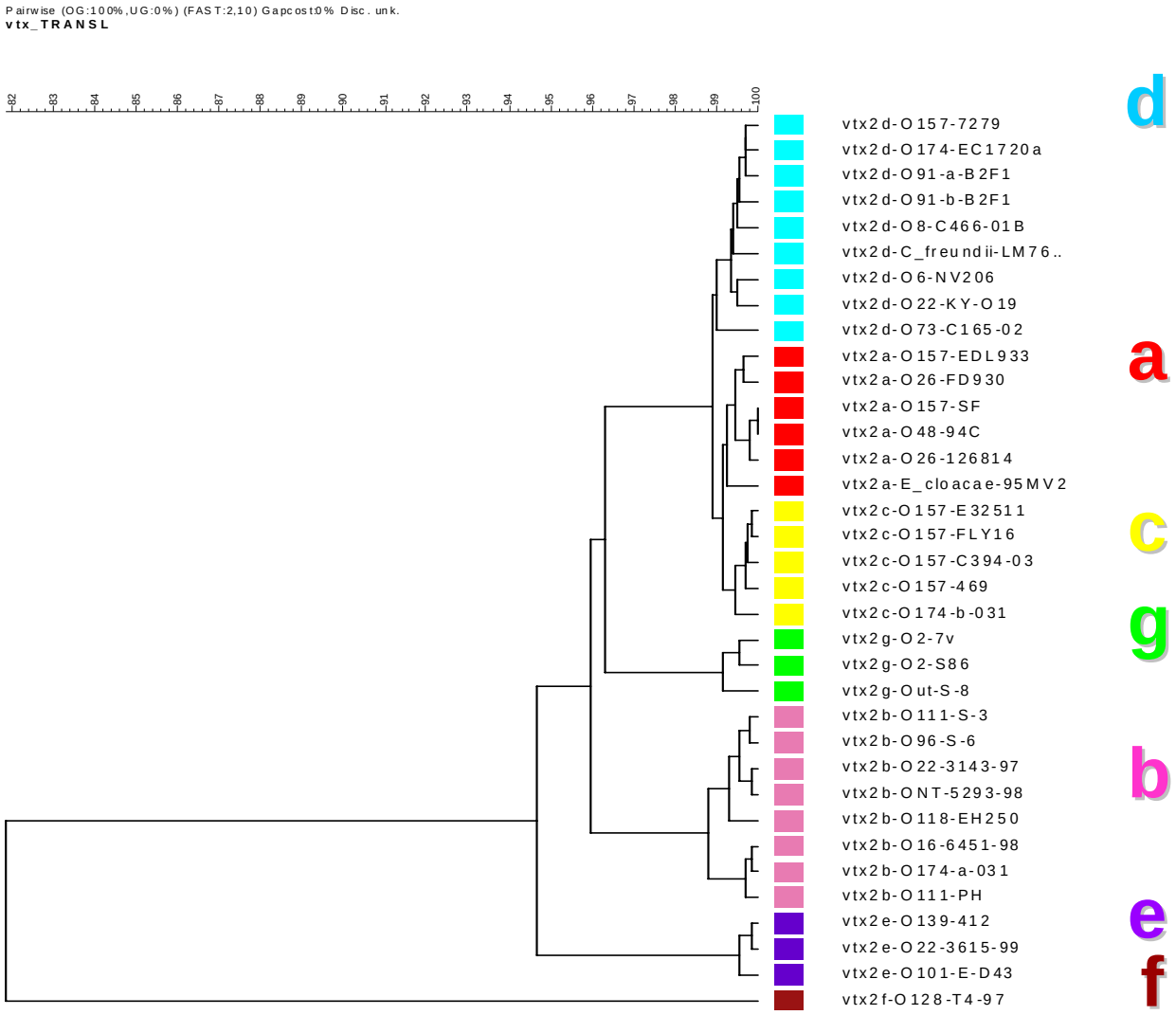


Figure 7: Stx2:7 subtypes (Stx2a-g); 35 variants
Source: F. Scheutz, USDA, FDA, CDC: Public non-O157 meeting, Washington DC 2007.



STEC Serotypes

Serotyping is an important basis for differentiating STEC and is often the starting point in the characterization of STEC strains. The serotype of an *E. coli* isolate is based on the O-antigen determined by the polysaccharide portion of cell wall lipopolysaccharide (LPS), and the H antigen due to flagella protein. There are 174 O-antigens and 53 H-antigens (Scheutz 2004) described so far in the international serotyping scheme, with *E. coli* isolates having various combinations of O and H antigens. A high percentage of STEC serotypes are nonmotile (NM) mutants of strains without an H antigen, but these strains are capable of causing illness as severe as STEC O157:H7 (Gyles 2006).

Severe disease and outbreaks are most commonly due to serotype O157:H7. Because of the importance of serotype O157:H7 in human disease and the ease in which STEC infections are detected and diagnosed, it is common to consider STEC serotypes in two major categories: O157 and non-O157. The most widely used methods for isolating STEC O157 are serotype and sorbitol fermentation specific and do not detect non-O157 strains. For this reason, the number of documented infections with STEC strains other than non-sorbitol-fermenting STEC O157 is underestimated (Strockbine 1998).

STEC strains are often considered as a group but there may be important differences between serotypes. Different serotypes may have differences in clinical features. Non-bloody diarrhea is more commonly reported among persons infected with non-O157 strains. Isolates from blood and urine are also more commonly seen among persons with non-O157 infections, therefore the spectrum of illness with non-O157 STEC may be wider than that for O157.

E. coli O157 seems to be the predominant serotype of STEC in the US, Canada, the UK, and Japan, but in continental Europe, Latin America, and Australia, non-O157s are much more common. However, the clinical presentation is not independently related to the serotype. Rather than the serotype or O group, the combined presence of the eae and Stx2 genes is an important predictor of HUS (Ethelberg 2004).

With the introduction of diagnostic methods targeting Stx or the Stx genes, more non-O157 infections are now being diagnosed in all countries, including the United States, Canada, the UK, and Japan. In Germany, where STEC infection is statutorily notifiable regardless of serotype, non-O157 STEC infections account for almost 80% of reported gastroenteritis cases, and approximately a third of STEC-associated HUS cases (Frank 2008). In addition to STEC O157:H7, other serotypes that have caused major outbreaks in the United States include O26:H11, O103:H2, O111:H-, O111:H8, O121:H-, and O145:H- (Bulte 2003). Table 2 provides the toxin profiles of fifteen of the most prevalent non-O157 STEC serotypes isolated from humans in the United States during 1983-2002.

Table 2: Toxin profiles and virulence factors of most prevalent non-O157 STEC serotypes isolated from humans, United States, 1983-2002. Source: Brooks et al. Journal of Infectious Diseases 2005: 192; 1422-1429

Serogroup	Age of donor, median (IQR), years[a]	Shiga toxin			*eae*	E-*hly*
		stx_1 alone	stx_2 alone	stx_1 and stx_2		
O26 (n = 209)	6 (2–18)	182/208 (88)	4/208 (2)	22/208 (11)	198/202 (98)	195/202 (96)
O111 (n = 152)	6 (2–21)	65/152 (43)	0/152 (0)	87/152 (57)	146/149 (98)	139/149 (93)
O103 (n = 117)	17 (3–31)	117/117 (100)	0/117 (0)	0/117 (0)	115/115 (100)	114/115 (99)
O121 (n = 80)	10 (4–34)	1/80 (1)	72/80 (90)	7/80 (9)	73/75 (97)	69/75 (92)
O45 (n = 63)	25 (13–48)	62/63 (98)	0/63 (0)	1/63 (2)	62/63 (98)	60/63 (95)
O145 (n = 43)	18 (6–42)	18/43 (42)	19/43 (44)	6/43 (14)	42/42 (100)	42/42 (100)
O165 (n = 14)	20 (8–66)	0/14 (0)	7/14 (50)	7/14 (50)	13/14 (93)	13/14 (93)
O118 (n = 9)	36 (2–52)	9/9 (100)	0/9 (0)	0/9 (0)	8/8 (100)	8/8 (100)
O91 (n = 8)	42 (30–46)	4/8 (50)	1/8 (12)	3/8 (38)	0/8 (0)	4/8 (50)
O113 (n = 8)	19 (10–41)	0/8 (0)	6/8 (75)	2/8 (25)	0/8 (0)	5/8 (62)
O153 (n = 7)	10 (9–11)	7/7 (100)	0/7 (0)	0/7 (0)	7/7 (100)	7/7 (100)
O146 (n = 6)	69 (64–74)	5/6 (83)	1/6 (17)	0/7 (0)	0/6 (0)	2/6 (33)
O174 (n = 6)	51 (21–75)	0/6 (0)	6/6 (100)	0/6 (0)	0/6 (0)	0/6 (0)
Other (n = 95)[b]	27 (7–62)	29/95 (30)	46/95 (48)	20/95 (20)	29/94 (31)	39/93 (42)
Undetermined (n = 123)	27 (7–62)	71/123 (58)	38/123 (31)	14/123 (11)	82/121 (68)	96/121 (79)
Total (n = 940)	15 (5–41)	570/939 (61)	200/939 (21)	169/939 (18)	775/918 (84)	793/917 (86)

NOTE. Data are proportion (%) of isolates, unless otherwise noted. IQR, interquartile range.

[a] For isolates for which the age of the person providing the specimen was included with submission to the CDC.

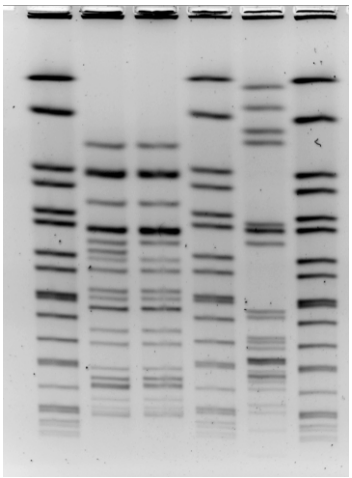
[b] For O22, O28, and O88, 5 isolates each (<1%); for O119, O128, and O172, 4 isolates each (<1%); for O6, O8, O60, O63, O104, O117, and O126, 3 isolates each (<1%); for O2, O14, O49, O50, O55, O73, O75, O96, O109, O110, O137, and O163, 2 isolates each (<1%); and for O1, O4, O5, O21, O38, O46, O48, O51, O77, O79, O83, O84, O86, O116, O124, O125, O140, O142, O143, O159, O168, O171, and O53/O117, 1 isolate each (<1%).

Molecular Subtyping and PulseNet

In order to investigate the relatedness of STEC strains isolated from outbreaks or sporadic cases, the gold standard method of strain typing that is used is pulsed-field gel electrophoresis (PFGE). PFGE is a Restriction Fragment Length Polymorphism (RFLP) method that uses restriction enzymes to generate a relatively small number of large DNA fragments. Such fragments are too large to separate by conventional electrophoresis but can be separated when subjected to a changing (pulsed) electrical field. The greatest difficulty in interpreting PFGE results comes in deciding whether or not patterns are indistinguishable. Such difficulties in interpreting subtyping results should serve as a

reminder that subtyping is an adjunct to, not a replacement for, a thorough epidemiological investigation (Strockbine 1998). For STEC O157 strains, restriction enzymes *Xba*I and *Bln*I are used as the primary and secondary enzymes, respectively (Figure 8).

Figure 8: PFGE Gel Image of STEC O157 Isolates Restricted with *Xba*I (lanes 2 and 3) and *Bln*I (lane 5); Molecular Size Standard *Salmonella* Braenderup in lanes 1, 4, and 6. Source: PulseNet *E. coli* national database, 2009



PFGE has worked effectively to identify STEC isolates from multiple cases that were epidemiologically related (Watanabe 2003). Subtyping is critical in linking cases in widely dispersed outbreaks. The role of subtyping is illustrated by the investigation of clusters of O157 infections in June and July 1997 that occurred hundreds of miles apart in Virginia and Michigan. Among the 70 ill persons identified, 97% had bloody diarrhea and 51% were hospitalized. Independent investigations linked both outbreaks to alfalfa sprouts. Strains from patients in the two states were compared and revealed indistinguishable PFGE patterns. Traceback of seeds from which the sprouts were grown revealed that they had only been shipped to these two states; all remaining seeds were removed from the market.

Subtyping has been an important adjunct to many other outbreaks of O157 (Griffin 1998). In the spinach outbreak in 2006, PFGE linked 183 cases in 26 states (CDC 2006). In an outbreak in 2007, PFGE linked 38 cases in 8 states to contaminated ground beef, resulting in a recall of 21.7 million pounds of ground beef products. Also in 2007, PFGE was used to link 21 cases in 10 states to frozen pepperoni pizza, resulting in a voluntary recall by the company. In 2008, PFGE was used to detect an outbreak involving 14 cases associated with a daycare center, 36 cases exposed to contaminated iceberg lettuce served at a university, an outbreak involving 17 cases associated with a college event, and an outbreak of 66 cases from 8 states associated with ground beef from a major supermarket chain, among many others.

To facilitate epidemiologic investigations, the Centers for Disease Control and Prevention (CDC), established a National Network for Molecular Subtyping for Foodborne Disease Surveillance called PulseNet in 1996. Since its inception in 1996, it has been instrumental in the detection, investigation, and control of numerous outbreaks caused by STEC O157:H7, *Salmonella enterica*, *Listeria monocytogenes*, *Shigella* spp., and *Campylobacter*. The PulseNet network is now being replicated in different ways in Canada, Europe, the Asia Pacific region, Latin America and the Caribbean, and the Middle East. These independent networks will allow public health officials to share molecular epidemiologic information in real-time, and will enable rapid recognition and investigation of multi-national foodborne disease outbreaks (Tauxe 2006).

Public health laboratories use standardized procedures for performance and interpretation of PFGE and share the data electronically. PFGE is used as the subtyping method in the network because it is accessible for many laboratories as well as being

highly discriminatory and reproducible for many pathogens. National databases at the CDC contain STEC O157 PFGE patterns, as well as other STEC serotypes, which public health laboratories can access to compare with local PFGE patterns (Swaminathan 2001, Gerner-Smidt 2006). Sharing of subtyping data on a national level has proved invaluable in determining the extent of foodborne outbreaks and identifying diffuse outbreaks that could not be detected by surveillance alone.

Electronic Foodborne Outbreaks Reporting System (eFORS)

Since 1973, the CDC has maintained a collaborative surveillance program for the collection and periodic reporting of data on the occurrence and causes of foodborne disease outbreaks (FBDOs) in the US. The Foodborne Disease Outbreak Surveillance System reviews data on FBDOs, defined as the occurrence of two or more cases of a similar illness resulting from the ingestion of a common food. State and local public health departments have primary responsibility for identifying and investigating FBDOs. State, local, and territorial health departments use a standard form to report these outbreaks to the CDC. A revised form became available in 1999. The revised form expanded the range of food items, places, and contributing factors that could be reported (Lynch 2006).

Since 2001, reports of FBDOs are submitted through a web application on the internet called the electronic Foodborne Outbreak Reporting System (eFORS). These reports summarize data collected with both the paper and web-based forms. The majority of forms are submitted by state, local, and territorial health departments, however, they can also be submitted by federal agencies and other sources. Reporting officials use

published criteria to determine whether a specific etiologic agent has been confirmed for an outbreak and submit reasons that reported food vehicles were implicated (Lynch 2006). In eFORS, data collection after 1998 is considered “Enhanced Surveillance”. Prior to 1998, about 500 outbreaks per year were reported, and after 1998, the average number of outbreaks reported increased to 1,250 outbreaks per year (Ayers 2008).

National Outbreak Reporting System (NORS)

In 2007, eFORS began undergoing developmental changes which led to the development of the National Outbreak Reporting System (NORS). NORS is being developed by the Division of Foodborne, Bacterial, and Mycotic Diseases (DFBMD) in collaboration with the Division of Parasitic Diseases, Division of Viral Diseases, Division of Viral Hepatitis, and National Center for Environmental Health within the CDC, and is expected to be deployed in 2009. This system will continue to monitor the overall burden and trends of foodborne diseases, as eFORS, but will integrate the reporting of foodborne, waterborne, zoonotic, and person-to person enteric disease outbreaks. NORS data will also be used for human illness attribution studies, which aim to attribute human cases of illness to specific sources, such as particular food commodities or animal reservoirs (Ayers 2008).

National Antimicrobial Resistance Monitoring System (NARMS)

The National Antimicrobial Resistance Monitoring System (NARMS) for enteric bacteria was established in 1996 and is a collaboration between the CDC and the USDA. Participating health departments forward every twentieth non-Typhi *Salmonella* isolate,

every *Salmonella* Typhi, every twentieth *Shigella* isolate, and every twentieth *E. coli* O157 isolate received at their public health laboratories to the CDC for antibiotic susceptibility testing. Because NARMS data have been collected continually since 1996, the data can be used to show trends that provide useful information about patterns of emerging resistance. Additionally, antimicrobial resistance data from humans provided by NARMS are important for the development of public health regulatory policy for the use of drugs in food-producing animals (CDC-NARMS 2008).

STEC in the Food Chain

An area of current exploration concerns how STEC enters the food chain. This begins with the ecology of STEC in animals and in the environment. An essential element to the full understanding of *E. coli* O157:H7 ecology is to determine whether cattle are typically transiently colonized (subsequently reinfected) or if the microbe is part of the normal flora and shed only periodically. It is likely that there are non-O157 strains that colonize cattle and do not cause disease in humans, but that cattle nonetheless are a significant reservoir for human pathogenic non-O157 STEC. One study suggests that living in a cattle-raising region appears to imply risk not only for STEC O157, but also for most non-O157 serogroups, and that cattle density is positively associated with overall STEC incidence (Frank 2008). In addition, some studies suggest that certain cattle, designated as “supershedders” have greater *E. coli* O157:H7 transmission potential than other cattle, whether through greater incidence or persistence of excretion, excretion of greater concentrations of *E. coli* O157:H7, or a combination of these factors (Cobbold 2007).

The USDA-FSIS and beef processing industry have implemented interventions to reduce contamination of ground beef, and the FDA has approved the use of irradiation of ground beef in the US. The success of this and other on-the-farm and slaughterhouse procedures offers promise for reducing meat-borne infections, but an even greater challenge is to prevent STEC contamination of water and vegetables.

Produce-associated outbreaks accounted for 21% of all foodborne outbreaks of *E. coli* O157:H7 from 1982 to 2002. Thirty outbreaks associated with leafy greens have been reported to the CDC through 2006. With more centralized production and wider distribution of produce including leafy greens, the propensity for large multistate outbreaks has increased. Such outbreaks have greatly influenced industry practices and FDA policy. However, for successful public health interventions to occur, mechanisms of produce contamination need to be successfully identified and understood (Sodha, 2008). Additionally, preventing foodborne disease depends in large part on engineering production systems for safety. Detecting and investigating outbreaks is an important way to determine the pathways that are most problematic (Tauxe 2006).

Conclusions

The study of the pathogenesis of STEC infections encompasses many different disciplines, including clinical microbiology, public health, diagnostics, animal ecology, and food safety, as well as cellular microbiology and the mechanisms of toxin action (Philpott 2003). This study will attempt to identify trends in the number of foodborne outbreaks caused by STEC O157 strains in the U.S. during 1999-2008 in addition to trends in the toxin profiles of those strains and HUS rates of STEC outbreaks. The number of STEC O157 isolates within the PulseNet national database and their

corresponding toxin profiles will be compared for each year to identify any existing trends. Two additional independent datasets, including a dataset collected from eFORS reports, and a dataset containing a collection of NARMS isolates will be examined for the same trends, in order to confirm trends observed in the PulseNet dataset. These trends will be compared to the HUS rates of recent STEC outbreaks in the United States, in an attempt to identify a direct correlation between an increase in Stx2-only producing strains and an increase in HUS rates.

CHAPTER III: METHODS

Institutional Review Board Application

The protocol title "Trends in Toxin Profiles of Human O157 Strains Using the PulseNet *E. coli* National Database and Electronic Foodborne Outbreak Reporting System (eFORS), 1999-2008" was approved by the Georgia State University Institutional Review Board on September 16, 2008. Protocol number is H09100.

Description of Datasets

An isolate is a sample of bacteria retrieved from an infected or contaminated source. Three independent datasets, each containing Shiga toxin-producing *E. coli* (STEC) isolates, were used for this study. These datasets include a collection of STEC isolates from within the PulseNet *E. coli* national database, a collection of STEC outbreaks from eFORS reports, and a random sample of isolates collected by the National Antimicrobial Resistance Monitoring System (NARMS).

PulseNet Dataset

The PulseNet *E. coli* national database contains PFGE profiles and toxin information for *E. coli* isolates of all serotypes from human and non-human sources. PulseNet participants (state, county, and city public health laboratories as well as federal food regulatory agency laboratories and the CDC) subtype all Shiga toxin-producing *E. coli* using at least the primary restriction enzyme (*Xba*I) immediately when they receive them from diagnostic laboratories. The PFGE profiles (DNA fingerprints) are then

uploaded to the PulseNet national database along with demographic information (age, gender, source type, geographic location) of the source. As of December, 2008, the PulseNet STEC database contained over 35,000 STEC isolates, however the dataset used in this study contains only human STEC O157 isolates from the USA, uploaded to the PulseNet database between 1999 and December 15, 2008, for which toxin information is known, which yielded a sample of 4,402 isolates.

The 4,402 isolates included in this dataset were categorized into three subsets according to their toxin profile (Stx1-only, Stx2-only, and Stx1+Stx2). The Stx1-only subset contained 69 isolates, the Stx2-only contained 2,057 isolates, and the Stx1+Stx2 subset contained 2,276 isolates. The number of isolates within each subset was compared for each year during the time period 1999 to 2008.

In the field of epidemiology, an outbreak is generally defined as the occurrence of disease that is greater than would otherwise be expected in a particular time and place. PulseNet identifies clusters, and a cluster is defined as a group of isolates with indistinguishable PFGE patterns limited in time and occurring at a frequency clearly above the historical baseline for that PFGE pattern. PulseNet clusters are communicated to state and CDC epidemiologists for investigation. All communication between PulseNet participants, epidemiologists, and other stake-holders in outbreak investigations related to PFGE patterns must be precise. Therefore, PulseNet database managers at the CDC assign a unique outbreak code to all clusters investigated. In this document, a PulseNet outbreak is defined as a cluster of isolates that has been given an outbreak code. The basic code is as follows: (YY)(MM)(LabID)(serotype code of the organism)-(number of cluster in the month). The first four digits of the outbreak code indicate the year and

month in which the cluster was detected. The LabID is the two-to four-letter PulseNet lab identifier for the laboratory that initially recognized the cluster (usually the state postal abbreviation code). The serotype code of the organism identifies which organism is involved in the cluster. The digit that follows the organism code denotes the number of cluster caused by that organism within that month and year in that state (Gerner-Smidt 2006). For example, the PulseNet-assigned outbreak code for the first outbreak of *E. coli* O157:H7 seen in January 2009 in Georgia would be 0901GAEXH-1. PulseNet began assigning outbreak codes in 2002.

Within the PulseNet dataset, toxin profiles were compared for each year of the time period 1999-2008 for all isolates in the dataset. Then, using the PulseNet-assigned outbreak code, outbreak isolates were separated from sporadic isolates to determine if trends were consistent. Those isolates given an outbreak code were considered as outbreak isolates and those isolates without outbreak codes were considered to be sporadic. Because outbreak codes were not used in PulseNet until 2002, outbreak and sporadic isolates were only separated for isolates occurring during 2002-2008.

In the PulseNet dataset, the outbreak code was also used to determine if there had been an increase in the number of STEC O157 outbreaks from the time period observed. Using the outbreak code, the number of outbreaks was calculated for each year, for the time period 2002-2008.

Age and Gender Trends in the PulseNet Dataset

When isolates of STEC O157 are submitted to the PulseNet national database, certain demographic information relating to the infected patient is linked to the PFGE

pattern, and both the PFGE pattern(s) and demographic information for an isolate are submitted to the national database as one entry. The demographic information that is submitted includes but is not limited to the patient's age, gender, source site (stool, blood, etc), and geographical location where the patient's specimen was collected. Patient and company names are not submitted to the PulseNet database to protect the privacy of those individuals and entities.

Using the PulseNet dataset, trends in age and gender distribution were examined in this study, to determine if certain toxin profiles are more predominantly seen among certain ages and genders in the population. To determine trends in age distribution, only isolates submitted with age information were included in this portion of the study. Likewise, to determine gender distributions, only isolates submitted with gender information (male or female) were included. The age and gender information was examined for each of the three toxin profile subsets (Stx1-only, Stx2-only, Stx1+Stx2) and trends were evaluated for the time period observed.

Seasonal and Geographic Trends in PulseNet Dataset

As previously mentioned, the geographical location where the patient's specimen was collected is submitted to the PulseNet national database, and this information may include the source country, source state, source county, and/or source city. Additionally, each isolate that is submitted to the PulseNet national database automatically receives a computer-generated upload date on the date that the isolate was submitted, and this upload date is linked to the isolate. In addition to the upload date, submitting public health laboratories may also submit an isolate date (typically the date the specimen was

received in the clinical laboratory), and a received date (the date the PFGE department in the public health laboratory received the isolate to perform PFGE on it). Either or both of these dates (isolate date and received date) may be submitted to the PulseNet national database when this information is available. An upload date is always available for every isolate, as it is a computer-generated date that automatically populates the database when isolates are submitted. In general, there is about one week between the isolate date and received date, and another week between the received date and upload date; i.e. the isolate date generally occurs about two weeks prior to the upload date.

In this study, each of the three subsets of toxin profiles were examined for geographical and seasonal distributions, using the source location (state) and upload date information available in the PulseNet database. Geographical distributions were mapped for each toxin profile on a template of the United States. One map was created for each of the three toxin profiles and showed the geographical distribution of all isolates with that toxin profile for 1999-2008. Seasonal distributions were identified by graphing the number of submissions and upload dates (using three-month intervals) for all isolates in each of the three toxin profile subsets.

Electronic Foodborne Outbreak Reporting System (eFORS) Dataset

The Electronic Foodborne Outbreak Reporting System (eFORS) provided a second dataset for this study. This dataset was compiled from reports of foodborne outbreaks and their implicated vehicles submitted to eFORS by state, local, and territorial health departments, as well as federal agencies. This dataset contained information for 233 STEC O157 outbreaks occurring in the US between 1998 and 2006 and was provided

by OutbreakNet, the network of epidemiologists at the CDC and in state laboratories working with foodborne infections. Information within this dataset included (when available) the reporting state, the estimated number of cases in each outbreak, transmission type (food, person-to-person, etc), implicated vehicle, number of HUS cases, number of hospitalized cases, and number of deaths.

HUS Rates in eFORS Dataset

HUS rates were known for 166 (71.2%) of the 233 outbreaks in the eFORS dataset and were compared for each year during 1998 to 2006 to determine trends. HUS rates were determined by dividing the number of HUS cases in the outbreak by the total number of cases in the outbreak in which HUS status was known. The average HUS rate for all outbreaks was calculated for each year.

Toxin information was only available for 43 (25.9%) of the 166 outbreaks with known HUS rates. Toxin information is not routinely reported in eFORS, therefore, the outbreaks in the eFORS dataset were matched to the PulseNet database using as much information as was available in the eFORS dataset in order to obtain the toxin profiles of the eFORS outbreaks. For those outbreaks in which an HUS rate and toxin profile was available, trends were noted to determine if higher HUS rates corresponded to a particular toxin type.

National Antimicrobial Resistance Monitoring System (NARMS) Dataset

NARMS data provided a third dataset for this study. When NARMS began surveillance in 1996, there were 14 participating health departments, known as “original

sites". In 2003, NARMS participation achieved national coverage with 54 sites. For this dataset, a simple random sampling scheme was devised, as follows:

1) From the original 14 NARMS sites, a simple random sample of 2 samples per site per year, (providing a total of 28 samples per year; encompassing 1996-2005) was sampled.

2) Starting with 2003, the year where NARMS increased to 54 sites, a simple random sample of 4 samples per site for 10 of the 54 sites (these 10 sites were selected randomly) per year, (providing an additional 40 samples per year; encompassing 2003-2005) was sampled.

The purpose of the random sampling to create the NARMS dataset was to confirm any trends seen in the PulseNet and eFORS datasets, by using a more random selection of isolates than in the latter two data sources. Additionally, NARMS data may be more representative of STEC O157 in the population, as PulseNet and eFORS are biased toward outbreak cases.

Toxin Types of Isolates within NARMS Dataset

The sum total of isolates in the NARMS dataset was 363 isolates. Random sampling of NARMS isolates was performed in order to confirm any trends seen in the PulseNet and eFORS datasets. For all NARMS isolates, toxin types were determined by PCR of the toxin genes for Stx1 and Stx2 using a published primer set (Paton 1998), and PFGE was performed. Both laboratory tests were performed at the CDC. Toxin profiles and PFGE patterns were evaluated to determine any trends during the time period 1996-2005.

Microbiological Methods: PCR Testing and PFGE Analysis

The PulseNet dataset included only isolates with known toxin information. To obtain toxin information, PCR analysis was performed and results were submitted to the PulseNet *E. coli* National Database by the submitting laboratory. PCR was also conducted by laboratorians at the CDC on isolates within the NARMS dataset, using the Paton primer set. This is a two-tiered approach to PCR analysis of fecal samples from patients with suspected STEC infection. Fecal culture extracts are initially screened for the presence of Stx genes using a pair of redundant oligonucleotide primers capable of detecting the amplification of a product from either Stx1 or Stx2 (including all known Stx2 subtypes associated with human disease). Any extracts yielding a positive result are subjected to a second round of analysis using two multiplex PCR assays, which provide confirmation of the presence of Stx genes (Paton A. 2003).

PFGE patterns submitted to the PulseNet national database by PulseNet certified laboratorians are prepared using a standardized protocol (Ribot 2006). In this procedure genomic DNA is prepared by embedding cells in agarose plugs and lysing the cells using lysozyme, sarcosyl, and deoxycholate with subsequent washes in a buffer solution. The DNA is digested in the agarose using the restriction enzyme *Xba*I. The plugs are placed in a 1.2% agarose gel. The restricted fragments are separated by PFGE using 0.5 X Tris-borated-EDTA buffer at 14 degree Celsius and Chef Dr III (Bio-Rad; Hercules, California, U.S.) gel apparatus. Conditions for electrophoresis is as follows: initial switch time, 2.2 seconds, final switch time, 63.8 seconds at an angle of 120 degrees at 6 Volts/centimeter for 20 hours. Restriction fragments are visualized using an ethidium bromide stain under ultra-violet light, and the PFGE pattern is photographed, digitized,

and saved as Tagged Image File Format (TIFF). These TIFFs are then analyzed using a customized software program called BioNumerics (Applied Maths, Saint-Martens Latem, Belgium).

By standardizing subtyping protocols and analysis tools, the patterns generated in the PulseNet network may be compared between laboratories. An essential feature of the PulseNet system is the use of a universal standard by all participants, which is run in every fourth to fifth lane in all gels, thus allowing for reliable normalization of the patterns of the isolates in the adjoining lanes. This standard, comprised of *Xba*I restriction fragments of *Salmonella* Braenderup strain H9812 is used as a reference DNA fragment size standard for all pathogens under surveillance in PulseNet (Hunter et al., 2005).

All PFGE profiles are assigned pattern names by PulseNet database managers. A PulseNet standardized pattern name consists of 11 characters in the format: XXXYYY.####. The first three characters (X) represent the organism (e.g., EXH is the code for STEC O157); the next three characters (Y) represent the restriction enzyme that was used to cut the DNA (e.g., X01 is the code that represents the enzyme *Xba*I); the four digits to the right of the decimal (#) are consecutive numbers assigned to new profiles as they are detected. This number ascends from 0001 and in no way indicates any kind of genetic relatedness between different patterns (Gerner-Smidt 2006).

Correlation of PFGE Patterns with Toxin Types

In the PulseNet dataset, isolates were analyzed to determine if certain PFGE patterns correlated with specific toxin profiles. The top 10 PFGE patterns (*Xba*I) in the *E. coli* national database were identified, based on their frequency of occurrence in the

national database between 1999 and 2008 (Table 3). These top 10 patterns were compared across each of three groups of isolates, (classified by their toxin profile as Stx1-only, Stx2-only, and Stx1+Stx2), to determine if any of the top 10 patterns correlated with a specific toxin profile.

Table 3: Top 10 PFGE patterns (*Xba*I) in the *E. coli* national database, based on their frequency of occurrence in the national database between 1999 and 2008. (Total *Xba*I patterns = 4,357)
Source: CDC PulseNet *E. coli* national database, 2008

PFGE-*Xba*I-pattern	Occurrence	Frequency
EXHX01.0047	517	11.90%
EXHX01.0074	202	4.60%
EXHX01.0200	152	3.50%
EXHX01.0224	150	3.40%
EXHX01.1343	131	3.00%
EXHX01.0124	121	2.80%
EXHX01.0125	114	2.60%
EXHX01.0087	102	2.30%
EXHX01.0008	102	2.30%
EXHX01.1486	84	1.90%

Correlation of Non-O157 STEC Serotypes and Toxin Types

In this study, the PulseNet dataset was comprised of STEC O157 isolates only. However, as of December 2008, approximately 18% of the PulseNet national database was comprised of non-O157 STEC isolates, and the number of non-O157 STEC isolates submitted to the database has increased tremendously in recent years as detection methods have changed in the clinical laboratories. All human non-O157 STEC isolates from the USA submitted to the PulseNet national database between 1999 and 2008 with

known toxin information were grouped into a separate subset of 1,422 isolates. The top six non-O157 serotypes were identified based on their frequency within this group of isolates. Toxin profiles were examined for the isolates belonging to the top six serotypes to determine if certain toxin profiles were more predominantly seen in common non-O157 STEC serotypes than in isolates of STEC O157.

Data Analysis

Data were analyzed in SAS 9.1 (SAS Institute, Inc., Cary, NC, USA). A Cochran-Armitage test was used to test for an increase in Stx2-only producing strains over time. Trend analyses were performed for all isolates in the PulseNet dataset, as well as separately for outbreak and sporadic isolates. Trend analyses were also performed for all isolates in the NARMS dataset. The null hypothesis was that the proportion of Stx2-only strains did not increase over time. Because the alternative hypothesis was that the proportion of Stx2-only isolates increased over time, the one-sided *p*-value is reported. The Wilcoxon Rank-sum test was used to assess differences in median age. For categorical variables (i.e., gender), differences were examined using a Chi-Square test.

CHAPTER IV: RESULTS

I. PulseNet Dataset

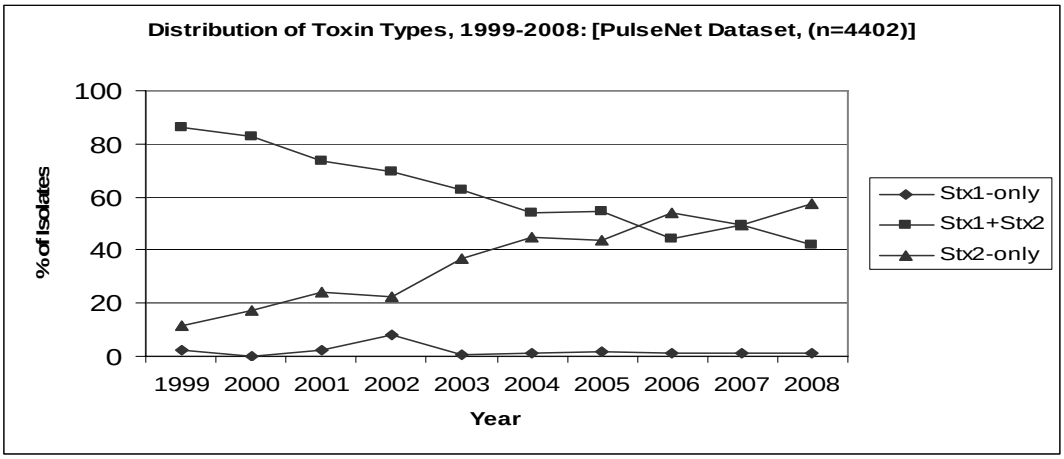
Distribution of Toxin Types: PulseNet Dataset

PFGE patterns of 4,402 STEC O157 isolates with known toxin information were submitted between 1999 and 2008 from public health laboratories and food regulatory agencies within the United States. Table 4 shows the number of isolates with each toxin type that was submitted per year. The percentage of isolates expressing Stx1-only was consistently low, and decreased from 2.3% in 1999 to 0.97% in 2008. The percentage of isolates expressing Stx1+Stx2 decreased from a high of 86.2% in 1999 to a low of 41.8% in 2008. The percentage of isolates expressing Stx2-only increased from a low of 11.5% in 1999 to a high of 57.2% in 2008 (Figure 9). (Cochran-Armitage trend test for increase in Stx2-only strains: Z=13.4; p<0.0001).

Table 4: Distribution of Toxin Types, 1999-2008: [PulseNet Dataset, (n=4402)]

	Isolates with Stx1-only (%)	Isolates with Stx1+Stx2 (%)	Isolates with Stx2-only (%)	Total
1999	2 (2.3%)	75 (86.2%)	10 (11.5%)	87
2000	0 (0.0%)	38 (82.6%)	8 (17.4%)	46
2001	3 (2.5%)	89 (73.6%)	29 (24.0%)	121
2002	19 (7.9%)	168 (69.4%)	55 (22.7%)	242
2003	1 (0.4%)	155 (62.8%)	91 (36.8%)	247
2004	5 (1.0%)	267 (54.3%)	220 (44.7%)	492
2005	9 (1.5%)	338 (54.6%)	272 (43.9%)	619
2006	11 (1.3%)	375 (44.5%)	456 (54.2%)	842
2007	10 (1.3%)	382 (49.2%)	384 (49.5%)	776
2008	9 (0.97%)	389 (49.8%)	532 (57.2%)	930
Total	**69**	**2276**	**2057**	**4402**

Figure 9: Distribution of Toxin Types (percentage of isolates), 1999-2008: [PulseNet Dataset, (n=4402)]
Cochran-Armitage trend test for an increase in Stx2-only strains: Z=13.4; p<0.0001



Distribution of Toxin Types: Sporadic vs. Outbreak-Related Isolates

The isolates within the PulseNet dataset were separated into outbreak-related isolates and sporadic isolates using the PulseNet-assigned outbreak code, to assess whether similar trends in toxin types existed among both outbreak and sporadic isolates. 2,519 isolates were classified as sporadic cases and 1,629 isolates were classified as outbreak-related cases. The distribution of toxin types was evaluated for both sets of isolates. Only isolates from 2002-2008 were included, as PulseNet did not utilize outbreak codes prior to 2002. The trends in toxin types for sporadic isolates are shown in Table 5 and Figure 10. The trends in toxin types for outbreak-related isolates are shown in tables 6 and Figure 11. (Cochran-Armitage trend test for increase in sporadic Stx2-only strains: Z=7.95; p<0.0001). (Cochran-Armitage trend test for increase in outbreak-related Stx2-only strains: Z=4.5; p<0.0001).

Table 5: Distribution of Toxin Types, Sporadic Isolates, 2002-2008: [PulseNet Dataset, (n=2519)]

	Isolates with Stx1-only (%)	Isolates with Stx1+Stx2 (%)	Isolates with Stx2-only (%)	Total
2002	15 (7.5%)	143 (71.5%)	43 (21.5%)	201
2003	1 (0.5%)	130 (63.1%)	75 (36.4%)	206
2004	5 (1.3%)	224 (59.4%)	148 (39.3%)	377
2005	9 (3.4%)	135 (50.6%)	123 (46.1%)	267
2006	9 (2.2%)	190 (45.8%)	216 (52.0%)	415
2007	9 (1.9%)	258 (53.4%)	216 (44.7%)	483
2008	9 (1.6%)	243 (42.6%)	318 (55.8%)	570
Total	**57**	**1323**	**1139**	**2519**

Figure 10: Distribution of Toxin Types, Sporadic Isolates (percentage of isolates), 2002-2008: [PulseNet Dataset, (n=2519)]
Cochran-Armitage trend test for an increase in Stx2-only strains: Z=7.9.5; p<0.0001

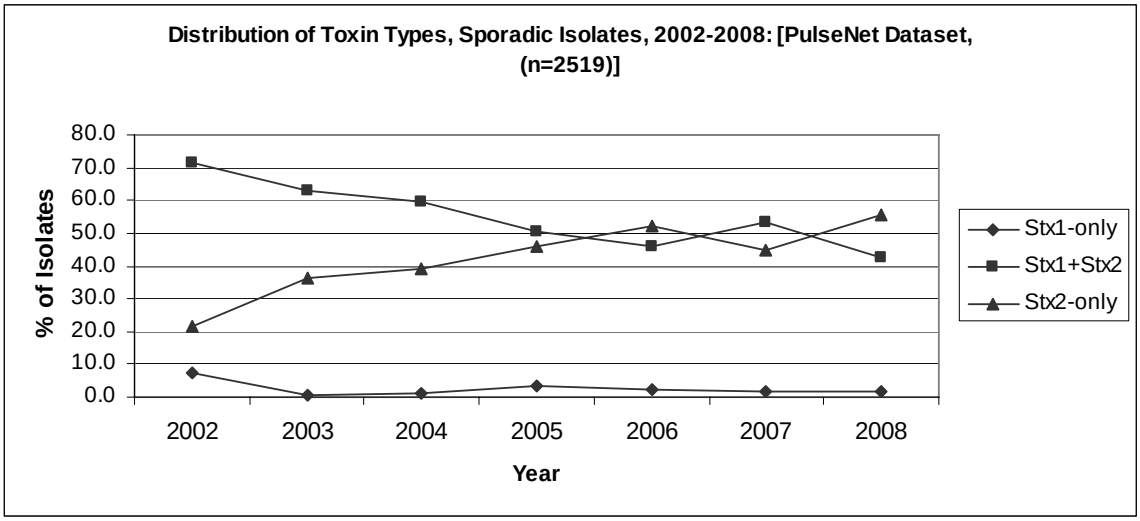
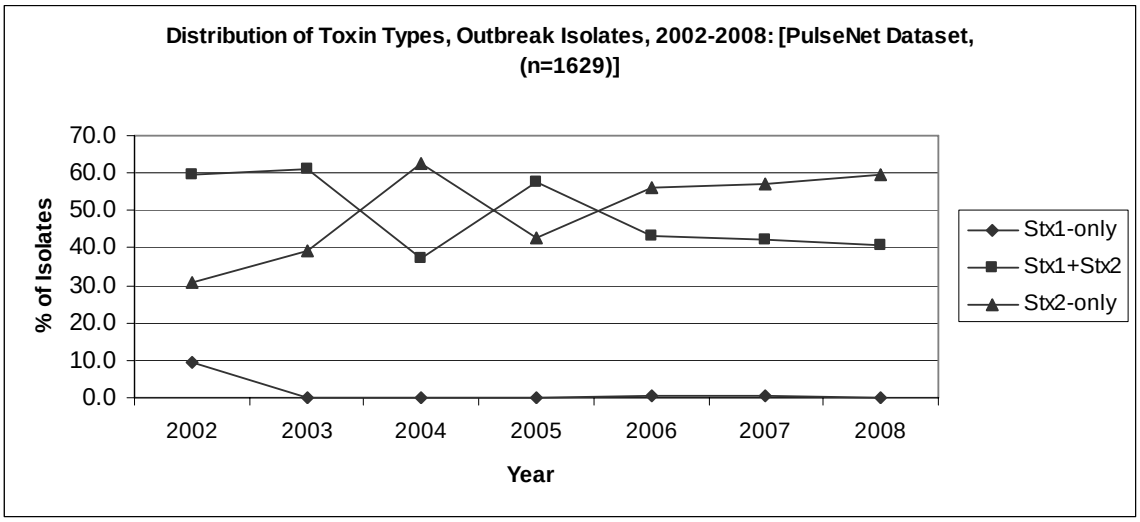


Table 6: Distribution of Toxin Types, Outbreak Isolates, 2002-2008: [PulseNet Dataset, (n=1629)]

	Isolates with Stx1-only (%)	Isolates with Stx1+Stx2 (%)	Isolates with Stx2-only (%)	Total
2002	4 (9.5%)	25 (59.5%)	13 (31.0%)	42
2003	0 (0.0%)	25 (61.0%)	16 (39.0%)	41
2004	0 (0.0%)	43 (37.4%)	72 (62.6%)	115
2005	0 (0.0%)	202 (57.5%)	149 (42.5%)	351
2006	2 (0.5%)	185 (43.3%)	240 (56.2%)	427
2007	1 (0.3%)	124 (42.3%)	168 (57.3%)	293
2008	0 (0.0%)	146 (40.6%)	214 (59.4%)	360
Total	**7**	**750**	**872**	**1629**

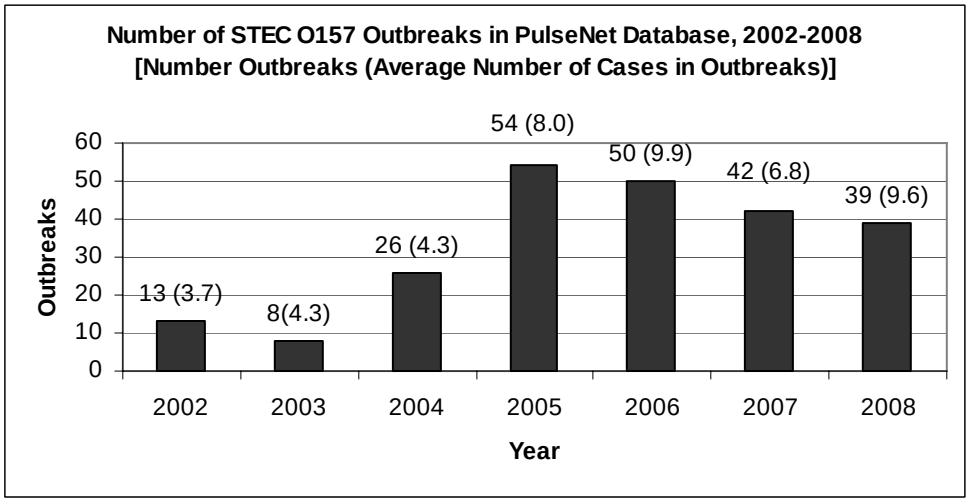
Figure 11: Distribution of Toxin Types, Outbreak Isolates (percentage of isolates), 2002-2008: [PulseNet Dataset, (n=1629)]
Cochran-Armitage trend test for an increase in Stx2-only strains: Z=4.5; p<0.0001



Number of Outbreaks within PulseNet Dataset

Using the PulseNet-assigned outbreak code, the number of outbreaks for each year was identified for the time period 2002-2008. The number of outbreaks was lowest in 2003, at 8 outbreaks, and highest in 2005, at 54 outbreaks. The mean number of outbreaks for all years was 33.1. The mean number of outbreaks for the first half of the time period (2002-2005) was almost half that of the second half of the time period (2005-2008), at 25.3 and 46.3 outbreaks, respectively. The number of outbreaks per year, as seen in the PulseNet database, is shown in Figure 12.

Figure 12: Number of STEC O157 Outbreaks per year, 2002-2008 and Average Number of Cases in Outbreaks (USA), 2002-2008 [PulseNet Dataset, (n=232 outbreaks)]



Age Distribution of Toxin Types

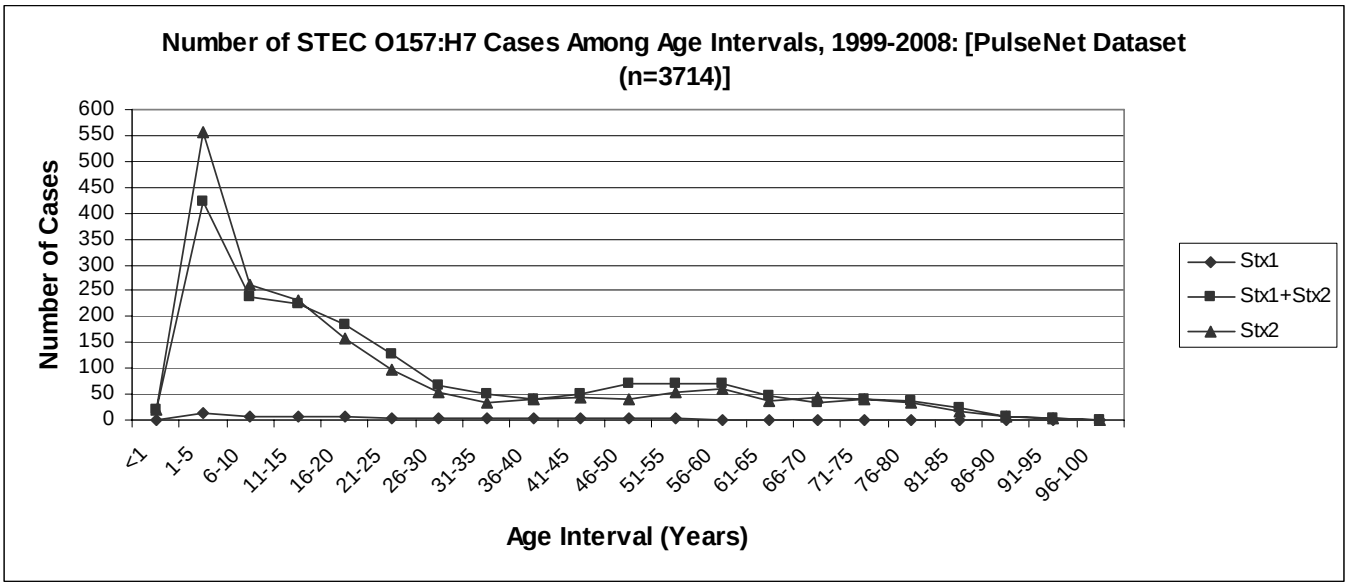
Age distributions of each toxin type were also examined using the PulseNet dataset. There were 3,714 human STEC O157 isolates with toxin and age information in the dataset. The median age was calculated for each of the toxin types (Stx1-only, Stx1+Stx2, and Stx2-only), for each year in the time period 1999-2008 (Tables 7-9). In 1999 and 2000, the median age for all isolates with Stx1-only was unknown, as there were no known ages for any isolates with Stx1-only in these years. The median age for all isolates with Stx1-only ranged from a low of 1 year in 2003 to a high of 32 years in 2006. The median age for all isolates with Stx1+Stx2 ranged from a low of 13 years in 2007 to a high of 21 years in 2000. The median age for all isolates with Stx2-only ranged from a low of 5 in 1999 to a high of 40 in 2000. There were no differences in the age distribution of patients infected with Stx1-only, Stx1+Stx2, or Stx2-only strains, and there was no change in the age distribution during the time period observed.

Differences in the median age for all years combined (1999-2008) were assessed for isolates with Stx1+Stx2 and Stx2-only using the Wilcoxon Two-Sample test. The median age for all isolates with Stx1+Stx2 was 24.3 years, and the median age for all isolates with Stx2-only was 21.4 years. This difference was found to be statistically significant (Z=4.9; p<0.0001).

The PulseNet dataset was used to identify if more cases were seen among young children relative to other ages during 1999-2008. The 3,714 isolates with toxin and age information were categorized into different age intervals, with five years per interval. Age intervals ranged from 1-5 years old to 96-100 years old. Results showed that the

highest number of cases was among the age interval 1-5 years, followed by 6-10 years and 11-15 years for each toxin type (Figure 13).

Figure 13: Number of STEC O157 Cases Among Age Intervals (USA), 1999-2008 [PulseNet Dataset, (n=3714)]



Gender Distribution of Toxin Types

3,645 human STEC O157 isolates in the PulseNet dataset contained toxin and gender information. To identify trends in gender distribution of toxin types using the PulseNet dataset, all human STEC O157 isolates with known toxin and gender information were classified into a group of 3,645 isolates. The percentage of female cases was calculated for each of the three toxin types, for each year in the time period 1999-2008 (Tables 7-9). In 1999 and 2000, the percentage of female cases for all isolates with Stx1-only was unknown, as there was no gender information available for any isolates

with Stx1-only in these years. There was also no gender information available for isolates with Stx2-only in 1999. The percentage of female cases for all isolates with Stx1-only ranged from a low of 50% in 2002 to a high of 66.7% in 2001, 2004, and 2005. The percentage of female cases for all isolates with Stx1+Stx2 ranged from a low of 46.2% in 2000 to a high of 62.0% in 2001. The percentage of female cases with Stx2-only ranged from a low of 47.8% in 2004 to a high of 65.4% in 2003. The average percentage of female cases for all isolates with Stx1-only, Stx1+Stx2, and Stx2-only (for all years in which gender information was available) was 57.9%, 53.7%, and 54.0%, respectively. Thus, the average percentage of female cases was above 50% for each of the toxin types for all years, indicating a slightly higher risk of infection among females. However, this difference was not found to be statistically significant as determined by a Chi-Square test (x^2=1.37; p=0.2426).

Table 7: Age and Gender Distribution (Age in Years) of Toxin Type Stx1-only, 1999-2008 (USA) [PulseNet Dataset, (age n=51; gender n=54)]

Stx1-only	Total Isolates with Gender Information	Age Distribution (Years)	Median	Gender Distribution
	N	(25%- 75% quartiles)	(Age in years)	Total Female (%)
1999	0	unknown	unknown	Unknown
2000	0	unknown	unknown	Unknown
2001	3	(6 - 28)	7	2 (66.7%)
2002	16	(12 - 31)	27	8 (50.0%)
2003	3	(1 - 1)	1	1 (33.3%)
2004	6	(8.5 - 33)	13	4 (66.7%)
2005	6	(2.25 - 12.75)	4.5	4 (66.7%)
2006	5	(15 - 46)	32	3 (60.0%)
2007	8	(6.5 - 29.25)	14	5 (63.0%)
2008	7	(4.25 - 17.75)	15.5	4 (57.0%)

Table 8: Age and Gender Distribution (Age in Years) of Toxin Type Stx1+Stx2, 1999-2008 (USA) [PulseNet Dataset, (age n=1844; gender n=1847)]

Stx1+Stx2	Total Isolates with Gender Information	Age Distribution (Years)	Median	Gender Distribution
	N	(25%- 75% quartiles)	(Age in Years)	Total Female (%)
1999	15	(3 - 56)	19	8 (53.3%)
2000	39	(8 - 46)	21	18 (46.2%)
2001	71	(7.5 - 45.25)	14	44 (62.0%)
2002	146	(6.75 - 42)	18	71 (48.6%)
2003	123	(6 - 51.75)	20.5	74 (60.2%)
2004	241	(7 - 44)	14	132 (54.8%)
2005	278	(6 - 47)	16	153 (55.0%)
2006	271	(4 - 26)	15	142 (52.4%)
2007	292	(5.5 - 34.5)	13	150 (51.4%)
2008	371	(5 - 32)	17	196 (52.8%)

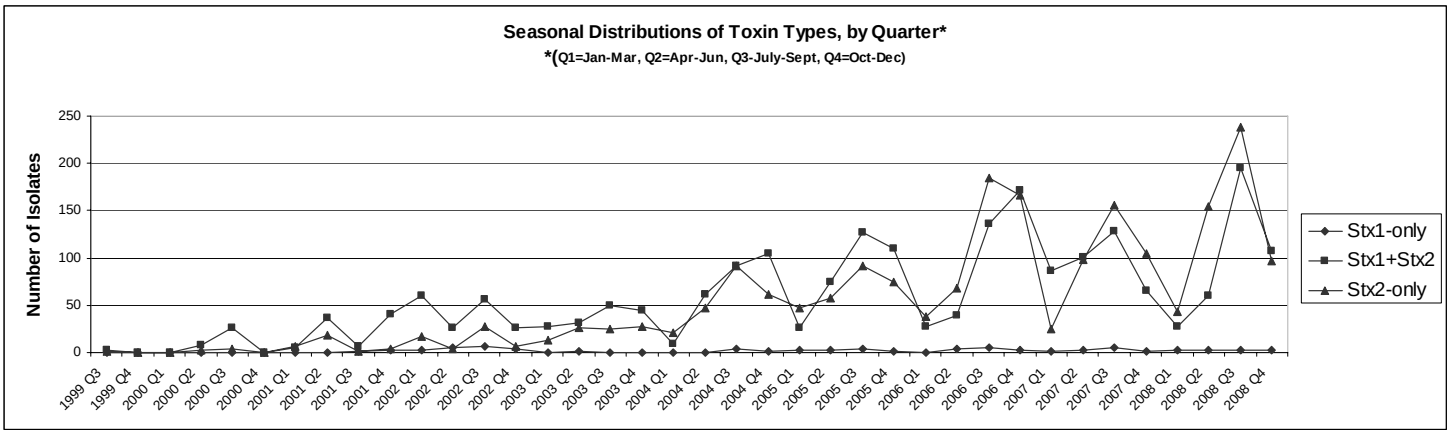
Table 9: Age and Gender Distribution (Age in Years) of Toxin Type Stx2-only, 1999-2008 (USA) [PulseNet Dataset, (age n=1819; gender n=1744)]

Stx2-only	Total Isolates with Gender Information	Age Distribution (Years)	Median	Gender Distribution
	N	(25%- 75% quartiles)	(Age in Years)	Total Female (%)
1999	0	(5 - 5)	5	Unknown
2000	8	(4.5 - 58)	40	4 (50.0%)
2001	23	(5 - 24.5)	7	13 (56.5%)
2002	46	(8 - 54)	20	29 (63.0%)
2003	78	(3.5 - 25)	13	51 (65.4%)
2004	180	(4 - 25)	12	86 (47.8%)
2005	208	(3 - 29.25)	9	104 (50.0%)
2006	356	(5 - 27.75)	13	187 (52.5%)
2007	349	(4 - 25)	12	185 (53.0%)
2008	496	(5 - 31)	13	240 (48.4%)

Seasonal Distribution of Toxin Types

Seasonal distributions of toxin types were also evaluated using the PulseNet dataset. All isolates within the dataset contained an upload date, which served as the approximate date in which STEC was isolated from the patient. Epidemiologists at the CDC use PulseNet upload dates to approximate isolation dates in outbreak investigations. The isolation date is generally estimated to be 14 days earlier than the upload date. Using the upload date, all isolates were divided into a year-quarter, with quarters making up a three-month time frame. For example, the first quarter of 2000 comprised all isolates with an upload date of January 1, 2000 to March 31st, 2000. The seasonal distribution of all isolates in the dataset ranged from the third quarter of 1999 (no isolates existed in the database for the 1st or 2nd quarter of 1999) to the 4th quarter of 2008. Each toxin type was evaluated separately (Figure 14). There was a general seasonal increase between the 2nd quarter (Q2) and 3rd quarter (Q3) for each toxin type within every year and a general seasonal decrease between Q3 and the 4th quarter (Q4) for each toxin type within every year. The number of isolates was consistently lowest during the first quarter (Q1) for each toxin type and year.

Figure 14: Seasonal Distributions of Toxin Types by Quarter, 1999-2008 (USA) [PulseNet Dataset, (n=4312)]



Geographic Distribution of Toxin Types

Geographical distributions of toxin types were also evaluated using the PulseNet dataset. 4,374 isolates within the dataset contained a source state, and source states were considered to be the state in which the patient became infected with STEC O157:H7. One map was created for each of the three toxin profiles and showed the geographical distribution of all isolates with that toxin profile from 1999-2008. Figure 15 shows the overall geographical distribution of Stx1-only strains for 1999-2008. Figure 15A and 15B show the geographical distribution of Stx1-only strains in 1999 compared to 2008. Figure 16 shows the overall geographical distribution of Stx1+Stx2 strains for 1999-2008. Figure 16A and 16B show the geographical distribution of Stx1+Stx2 strains in 1999 compared to 2008. Figure 17 shows the overall geographical distribution of Stx2-only strains for 1999-2008. Figure 17A and 17B show the geographical distribution of Stx2-only strains in 1999 compared to 2008. There were no clear differences between regions,

except that CA, MI, OH, and VA appeared to have the highest numbers of submissions for each of the toxin profiles. For isolates with Stx2-only, TX also appeared to have a higher number of submissions relative to other states.

Figure 15: Overall Geographic Distribution of Isolates with Stx1-only, 1999-2008 (USA) [PulseNet Dataset, (n=67)]

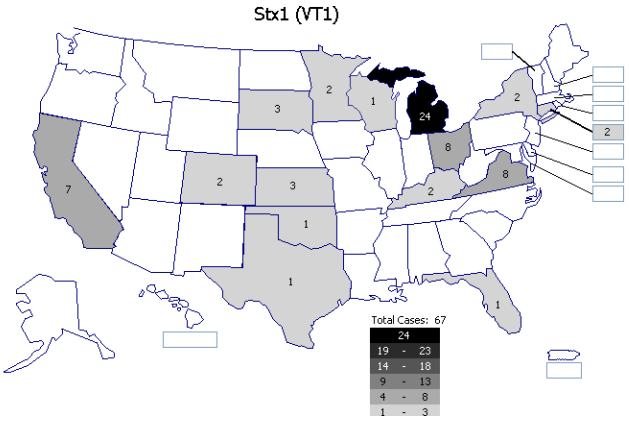
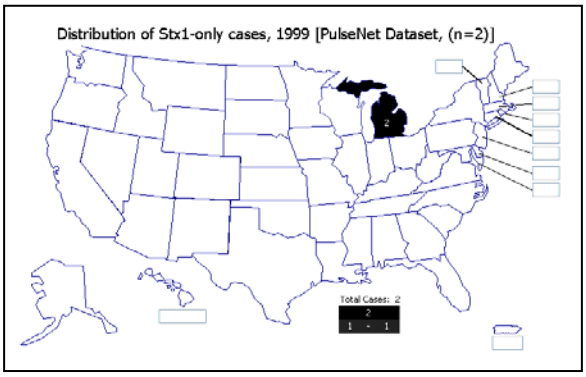


Figure 15A and 16B: Geographic Distribution of Isolates with Stx1-only, 1999 and 2008 (USA) [PulseNet Dataset, (1999 n=2; 2008 n=9)]



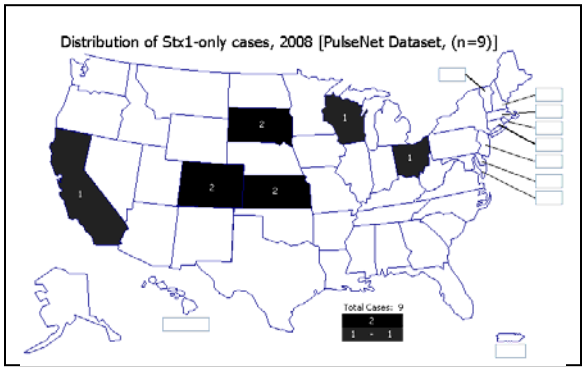


Figure 16: Overall Geographic Distribution of Isolates with Stx1+Stx2, 1999-2008 (USA) [PulseNet Dataset, (n=2265)]

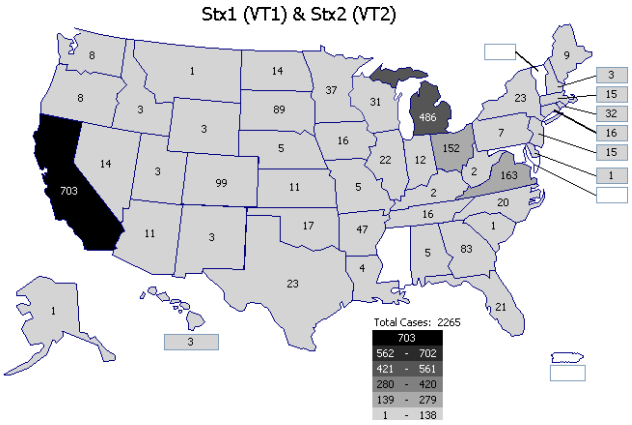
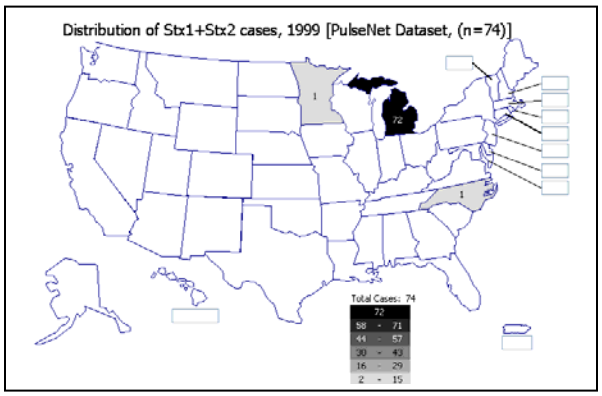


Figure 16A and 16B: Geographic Distribution of Isolates with Stx1+Stx2, 1999 and 2008 (USA) [PulseNet Dataset, (1999 n=74; 2008 n=389)]



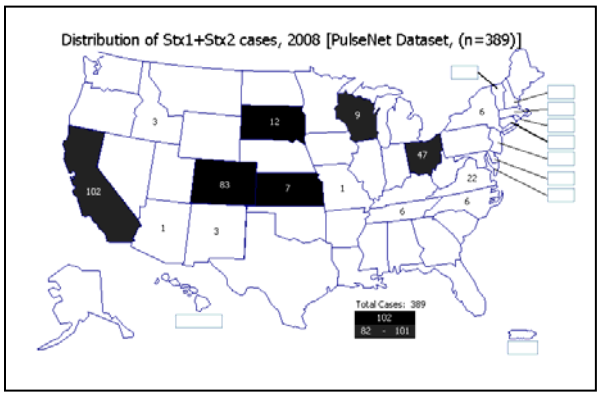


Figure 17: Overall Geographic Distribution of Isolates with Stx2-only, 1999-2008 (USA) [PulseNet Dataset, (n=2042)]

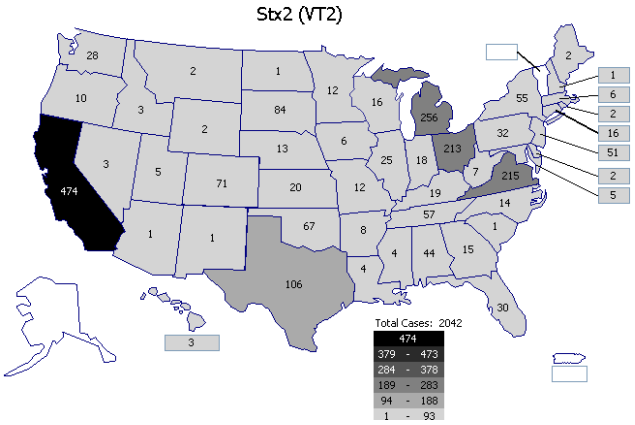
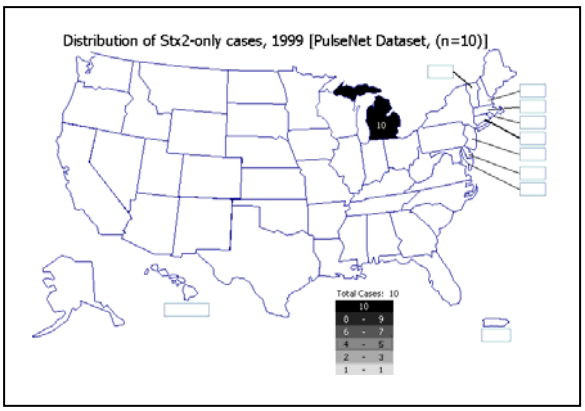
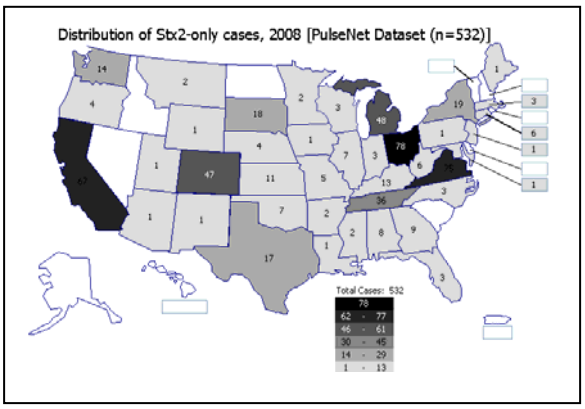


Figure 17A and 17B: Geographic Distribution of Isolates with Stx2-only, 1999 and 2008 (USA) [PulseNet Dataset, (1999 n=10; 2008 n=532)]





Distribution of Toxin Types by PFGE

The PulseNet dataset was also used to identify if certain toxin types were associated with particular PFGE patterns. The PulseNet-designated PFGE pattern name was used to make this assessment. The top ten *Xba*I patterns were identified based on their frequency of occurrence in the PulseNet dataset between 1999 and 2008. The percentage of isolates with each toxin type for each of the top ten patterns is shown in

Figure 18. Figures 19 and 20 show the percentage of isolates with these ten patterns from 1999-2003 and 2004-2008, respectively. For all patterns except EXHX01.1343 and EXHX01.0008, the percentage of Stx2-only strains increased from 1999-2003 to 2004-2008. One hundred percent of isolates with pattern EXHX01.1486 had toxin type Stx2-only. For patterns EXHX01.0047, EXHX01.0200, EXHX01.0224, EXHX01.0124, EXHX01.0125, there was a strong association between the toxin profile and PFGE pattern, as more than 90% of isolates with these patterns were associated Stx2-only. For patterns EXHX01.0074, EXHX01.1343, EXHX01.0087, and EXHX01.0008, more than 80% of the isolates were associated with Stx1+Stx2. None of the patterns within the top ten were associated with Stx1-only. Therefore, there seems to be an association between PFGE pattern and toxin profile for most patterns, although this association is not absolute.

Additionally, the top five PFGE patterns of isolates with Stx1-only were identified and were found to be EXHX01.0074, EXHX01.0079, EXHX01.0087, EXHX01.3417, and EXHX01.3138. When compared against the entire *E. coli* national database, the latter two PFGE patterns were only seen with toxin type Stx1-only. However, each of these patterns had only been seen twice in the entire database, so there was not a large enough sample to predict that these two patterns are exclusively associated with Stx1-only producing strains.

Figure 18: Top 10 *E. coli* PFGE patterns (*Xba*I) and their Toxin Types (USA), 1999-2008, [PulseNet Dataset, (n=1676)]

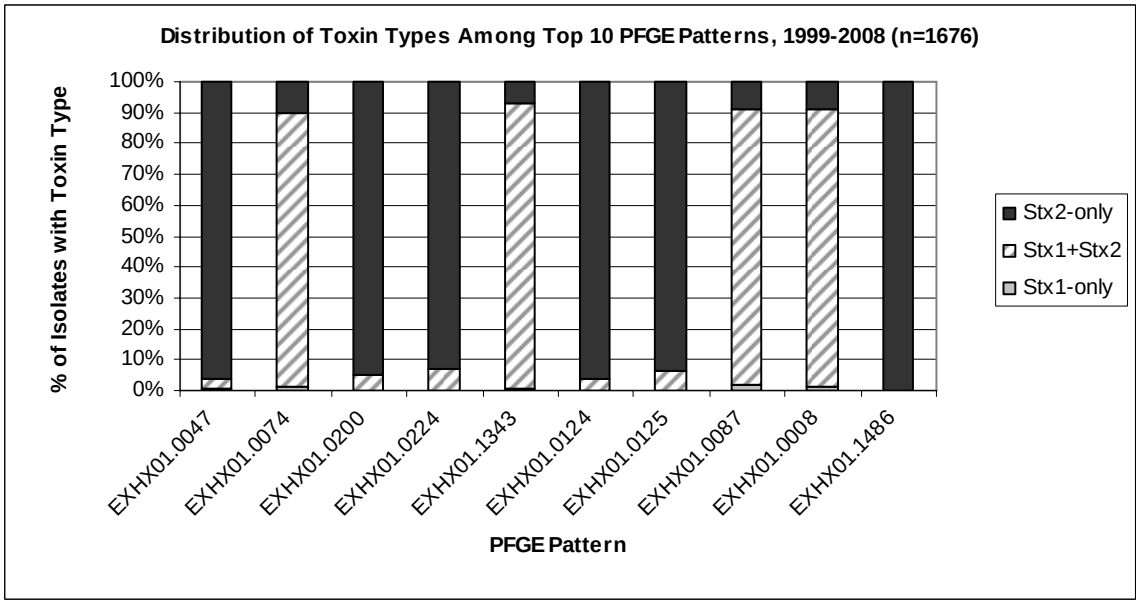


Figure 19: Top 10 *E. coli* PFGE patterns (*Xba*I) and their Toxin Types (USA), 1999-2003, [PulseNet Dataset, (n=188)]

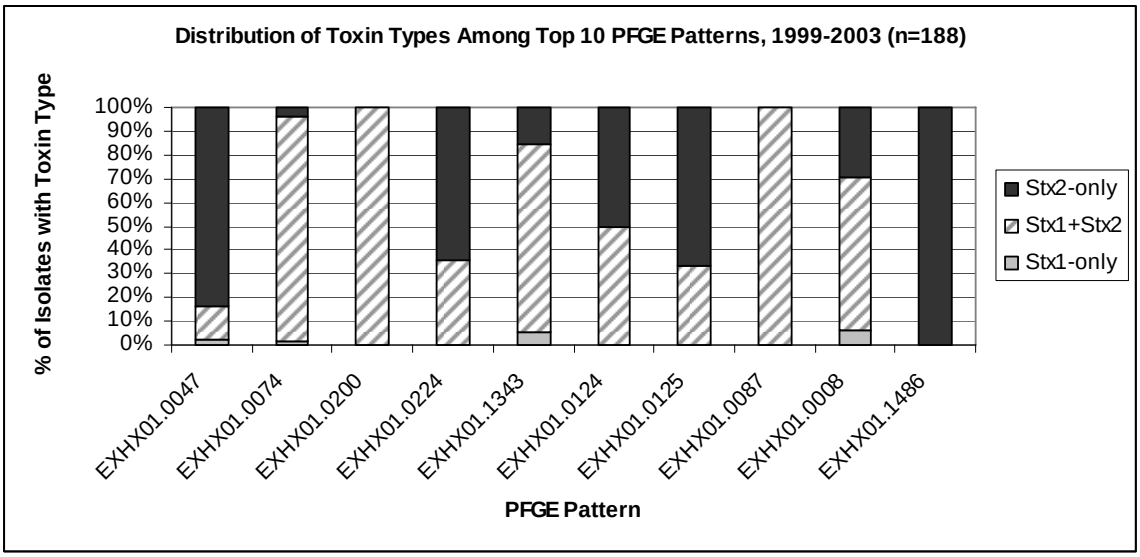
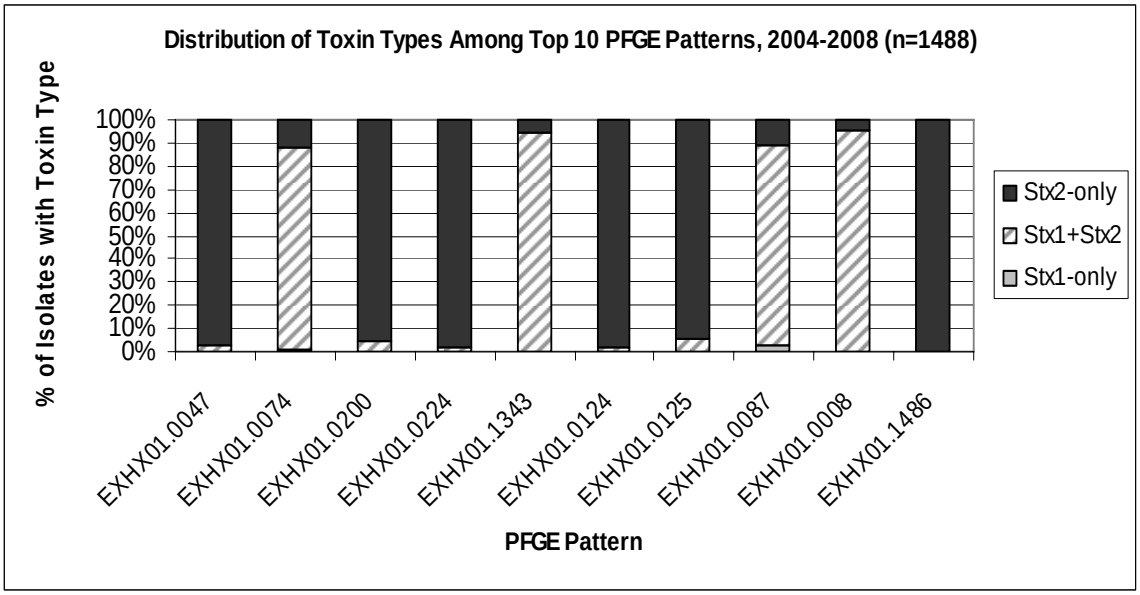


Figure 20: Top 10 *E. coli* PFGE patterns (*Xba*I) and their Toxin Types (USA), 2004-2008, [PulseNet Dataset, (n=1488)



Distribution of Toxin Types among Non-O157 STEC Isolates

The PulseNet database was also used to evaluate whether certain toxin profiles were seen at higher frequencies among non-O157 STEC serotypes. The top six non-O157 STEC serotypes were identified based on their frequency in the PulseNet national database for the time period 1999-2008 (Table 10). The percentage of isolates with each toxin type was identified for each of the top 6 non-O157 serotypes. For all serotypes, with the exception *E. coli* O121, the percentage of isolates with Stx1-only was higher than the percentage of isolates with Stx1+Stx2 or Stx2-only (Figure 21). The distribution of toxin types among isolates with *E. coli* O121 is shown in table 11.

Table 10: Number of Isolates with Top 6 Non-O157 STEC Serotypes (USA), 1999-2008 [PulseNet Dataset, (n=1422)]

Serotype	Occurrence	Frequency (n=1422)
E. coli O26	249	17.5%
E. coli O111	207	14.6%
E. coli O103	157	11.0%
E. coli O121	101	7.1%
E. coli O45	91	6.4%
E. coli O145	39	2.7%

Figure 21: Distribution of Toxin Types among Top 6 Non-O157 STEC Serotypes (USA), 1999-2008 [PulseNet Dataset, (n=1422)]

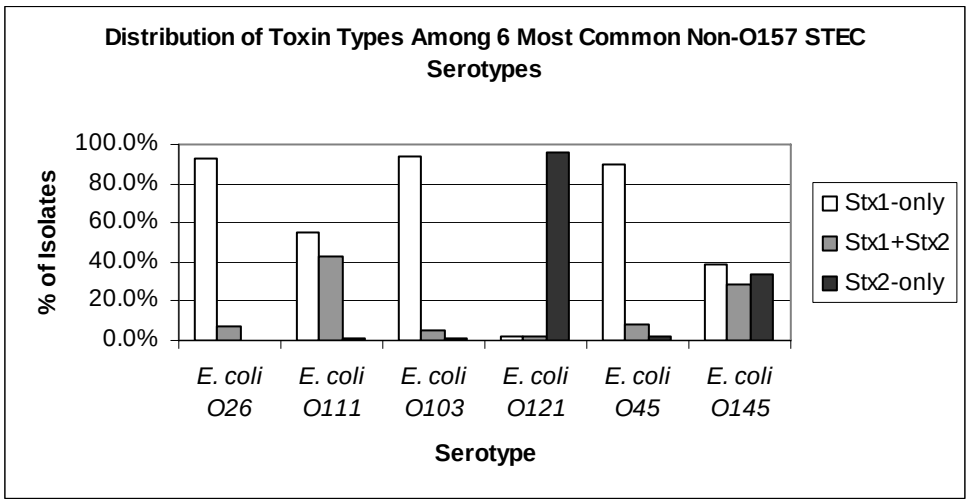


Table 11: Distribution of Toxin Types among *E. coli* O121 isolates (USA), 2003-2008* [PulseNet Dataset, (n=101)]

	Stx1-only	Stx1+Stx2	Stx2-only
2003	0	0	6
2004	1	0	8
2005	0	0	4
2006	1	1	14
2007	0	0	47**
2008	0	1	18

*No *E. coli* O121 isolates with known toxin information were submitted to the PulseNet database prior to 2003

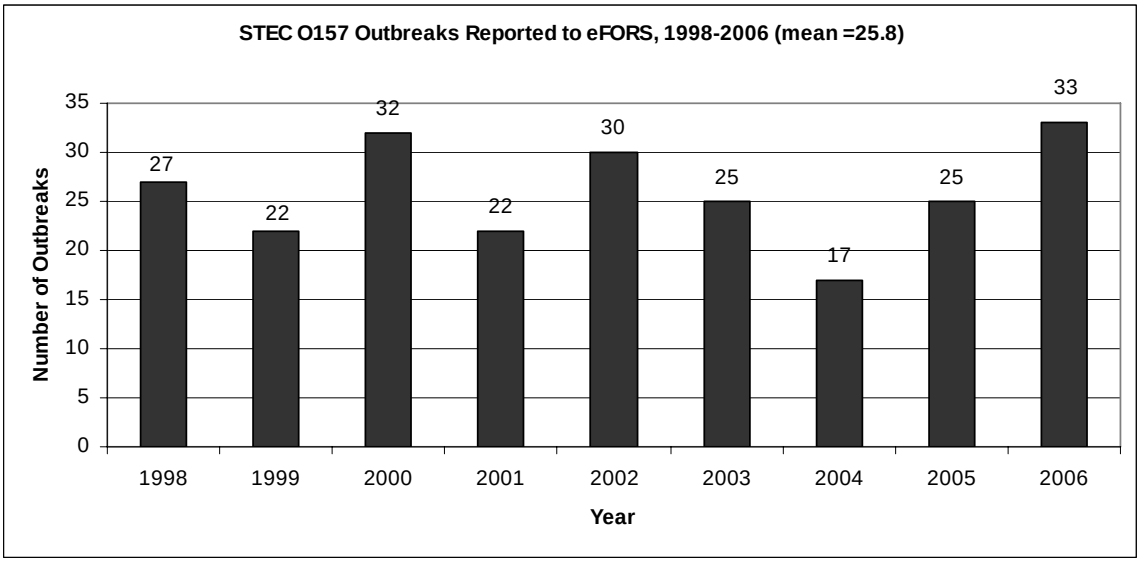
**Number of isolates in 2007 with Stx2-only was elevated due to an outbreak in a prison

II. eFORS Dataset

Number of Outbreaks Within eFORS Dataset

The number of STEC outbreaks reported to eFORS was identified for the years 1998-2006 (n=233) and is shown in figure 22. There were no observable trends seen in the number of outbreaks during this time period. The number of outbreaks ranged from a low of 17 outbreaks in 2004 to a high of 33 outbreaks in 2006. The average number of outbreaks for all years was 25.8.

Figure 22: Number of STEC O157 Outbreaks Reported to eFORS, 1998-2006 [eFORS Dataset, (n=233 outbreaks)]



HUS Rates of Outbreaks and Toxin Types

HUS rates were available for 166 (71.2%) of the 233 outbreaks in the eFORS dataset and were compared for each year during 1998 to 2006 to determine trends. HUS rates were calculated by dividing the number of HUS cases in the outbreak by the number of cases in the outbreak in which an HUS status was known. The number of outbreaks in the eFORS dataset with known HUS rates ranged from a low of 6 in 1998 to a high of 31 in 2006 (Table 12). The average HUS rate was determined for all outbreaks occurring in each year (Figure 23). The average HUS rate ranged from a low of 0.40% in 1998 to a high of 16.1% in 2006.

Toxin information was only available for 43 (25.9%) of the 166 outbreaks with known HUS rates. No toxin information was available for any outbreaks occurring prior to 2000. Three outbreaks were caused by Stx1-only producing strains; one in 2000 (HUS rate = 45.5%), one in 2002 (HUS rate unknown), and one in 2005 (HUS rate = 0.0%).

Seventeen outbreaks occurring between 2001 and 2006 were caused by Stx1+Stx2-producing strains, and HUS rates for these outbreaks ranged from 0.0% to 50.0% (the outbreak with an HUS rate of 50.0% only contained three cases, of which two had an HUS status, one positive and one negative). There were 23 outbreaks with toxin type Stx2-only which were exclusively seen in 2005 and 2006, with the exception of two that occurred in 2003. The HUS rates for these outbreaks ranged from 0.0% to 50.0% (the outbreak with an HUS rate of 50.0% only contained 8 cases, of which all had an HUS status, 4 positive and 4 negative). These results are shown in table 13.

Table 12: Number of STEC O157 Outbreaks Submitted to eFORS with Available HUS Rates, 1998-2006 [eFORS Dataset, (n=166)]

Year	Number of Outbreaks Submitted with HUS Rates
1998	6
1999	14
2000	22
2001	14
2002	20
2003	18
2004	16
2005	25
2006	31

Figure 23: Average HUS Rates of STEC O157:H7 Outbreaks Submitted to eFORS, 1998-2006 [eFORS Dataset, (n=166)]

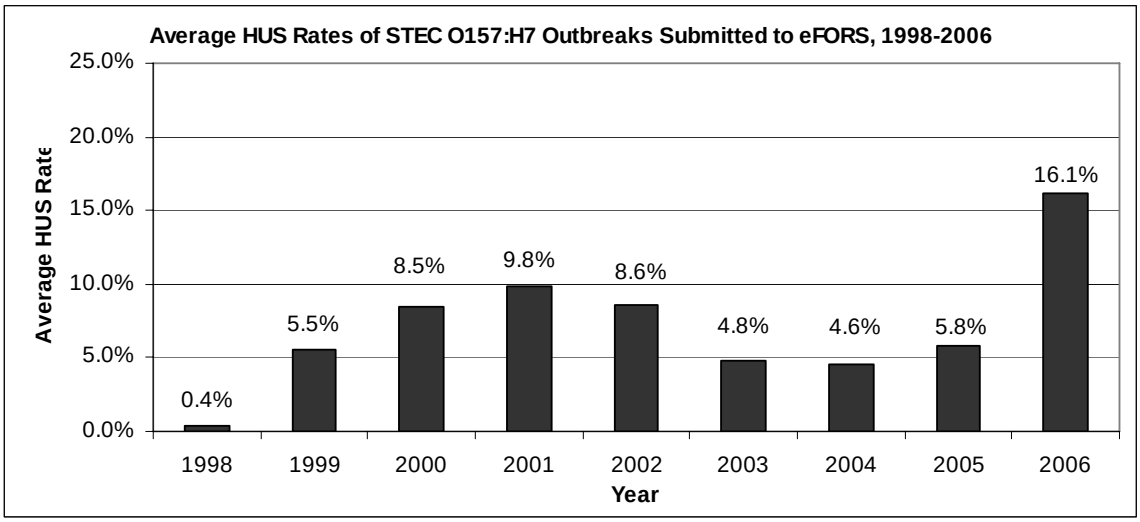


Table 13: HUS Rates of STEC O157:H7 Outbreaks Submitted to eFORS with Available Toxin Information, 1998-2006 [eFORS Dataset, (n=43)]

Year of Outbreak	Month of Outbreak	Estimated Total Cases	Cases with Known HUS Information	HUS Cases	HUS Rate	Toxin
2000	Apr	15	11	5	45.5%	stx1*
2001	Oct	28	28	0	0.0%	stx1+2
2002	Aug	74	unknown	1	unknown	stx1
2002	Aug	16	unknown	unknown	unknown	stx1+2
2003	Aug	18	6	2	33.3%	stx1+2
2003	Apr	13	unknown	3	unknown	stx2
2003	Oct	16	unknown	unknown	unknown	stx2
2004	Apr	59	29	7	24.1%	stx1+2
2004	May	4	4	0	0.0%	stx1+2
2004	Nov	6	6	0	0.0%	stx1+2
2005	Aug	52	52	0	0.0%	stx1
2005	May	3	3	0	0.0%	stx1+2
2005	June	8	8	0	0.0%	stx1+2
2005	Aug	18	18	2	11.1%	stx1+2
2005	Sept	34	30	2	6.7%	stx1+2
2005	Oct	64	64	0	0.0%	stx1+2
2005	Oct	12	12	0	0.0%	stx1+2
2005	Jan	3	unknown	unknown	unknown	stx2
2005	Jan	2	2	0	0.0%	stx2
2005	Apr	60	60	8	13.3%	stx2
2005	Aug	6	6	0	0.0%	stx2
2005	Aug	5	5	0	0.0%	stx2
2005	Sept	14	14	1	7.1%	stx2
2005	Oct	4	4	0	0.0%	stx2
2005	Oct	9	7	0	0.0%	stx2
2005	Oct	3	3	0	0.0%	stx2
2005	Nov	18	15	3	20.0%	stx2
2006	Jan	2	2	0	0.0%	stx1+2
2006	Mar	2	2	0	0.0%	stx1+2
2006	May	3	2	1	50.0%	stx1+2
2006	Aug	5	5	0	0.0%	stx1+2
2006	Sept	6	6	2	33.3%	stx1+2
2006	Mar	6	6	1	16.7%	stx2
2006	Apr	7	7	1	14.3%	stx2
2006	May	4	4	1	25.0%	stx2
2006	June	5	5	1	20.0%	stx2
2006	June	3	3	1	33.3%	stx2
2006	June	2	2	0	0.0%	stx2
2006	Aug	4	4	1	25.0%	stx2
2006	Aug	8	8	4	50.0%	stx2
2006	Nov	3	3	0	0.0%	stx2
2006	Dec	21	21	0	0.0%	stx2
2006	Aug	3	3	1	33.3%	stx2

* It is possible that the strain causing this outbreak originated with Stx1+Stx2, but subsequently lost the phage that produces the Stx2 toxin gene.

III. NARMS Dataset

Distribution of Toxin Types within NARMS Dataset

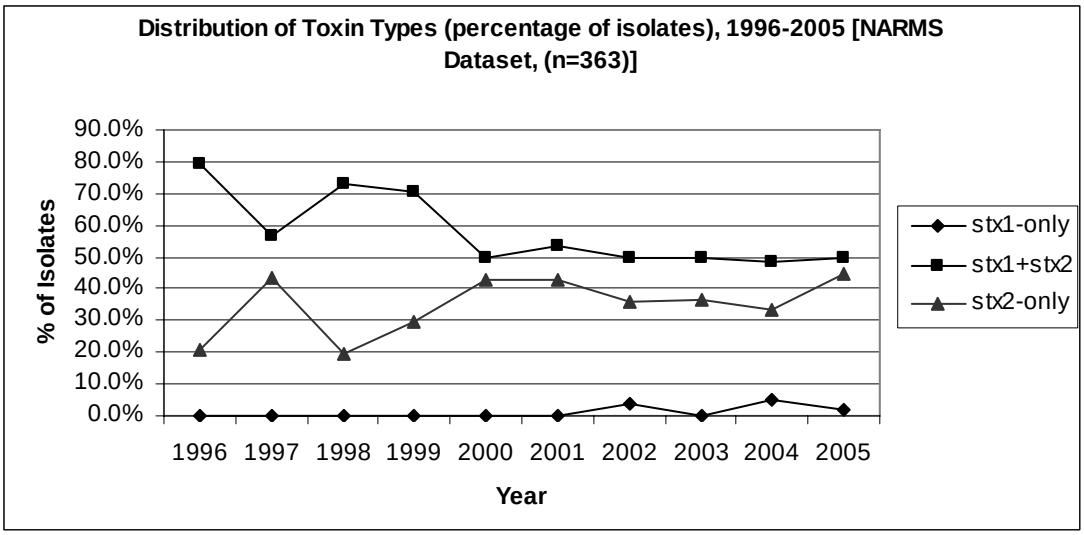
The NARMS dataset was used to identify trends in toxin profiles for the time period 1996-2005. The number and percentage of isolates with each toxin type was calculated for each year and are shown in table 14 and figure 24, respectively. The percentage of isolates with Stx1-only remained very low in all years. The percentage of isolates with Stx1+Stx2 decreased from a high of 79.2% in 1996 to 50.0% in 2005. The percentage of isolates with Stx2-only increased from 20.8% in 1996 to a high of 45.0% in 2005. Cochran-Armitage trend test for an increase in Stx2-only strains: $Z=2.32$; $p<0.0101$.

In all years except for 1996, 1997, and 1999, there was a small percentage of isolates in which the PCR reaction failed due to no amplification (na). These isolates were repeated with a different PCR assay, which differed from the original assay by targeting slightly different regions of the Stx genes. Some isolates still revealed no amplification, which may have been due to mutations in the toxin genes, or the organism being non-toxigenic O157, which would have led to the toxin genes being absent or too broken to be amplified.

Table 14: Number of Isolates with each Toxin Type (na: no amplification), 1996-2005 [NARMS Dataset, (n=363)]

	Isolates with Stx1-only	Isolates with Stx1+stx2	Isolates with Stx2-only	na	Total
1996	0 (0.0%)	19 (79.2%)	5 (20.8%)	0 (0.0%)	24
1997	0 (0.0%)	13 (56.5%)	10 (53.5%)	0 (0.0%)	23
1998	0 (0.0%)	19 (73.1%)	5 (19.2%)	2 (7.7%)	26
1999	0 (0.0%)	19 (70.4%)	8 (29.6%)	0 (0.0%)	27
2000	0 (0.0%)	14 (50.0%)	12 (43.0%)	2 (7.1%)	28
2001	0 (0.0%)	15 (53.6%)	12 (42.9%)	1 (3.6%)	28
2002	1 (3.6%)	14 (50.0%)	10 (35.7%)	3 (10.7%)	28
2003	0 (0.0%)	29 (50.0%)	21 (36.2%)	8 (13.8%)	58
2004	3 (5.0%)	29 (48.3%)	21 (34.4%)	8 (13.3%)	61
2005	1 (1.7%)	30 (50.0%)	27 (45.0%)	2 (3.3%)	60
Total	**5**	**201**	**131**	**26**	**363**

Figure 24: Distribution of Toxin Types (percentage of isolates), 1996-2005 [NARMS Dataset, (n=363)]
Cochran-Armitage trend test for an increase in Stx2-only strains: Z=2.32; p<0.0101



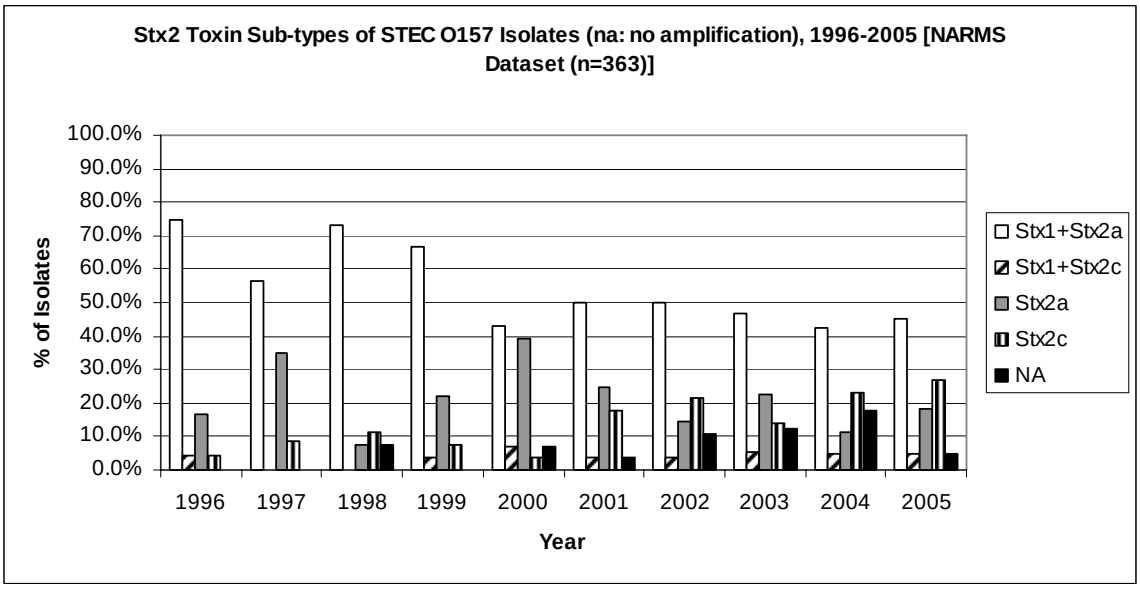
Stx2 Toxin Subtypes within NARMS Dataset

Stx2 toxin subtypes (a,c) were evaluated for all isolates in the NARMS dataset with either toxin type Stx1+Stx2 or Stx2-only. The percentage of isolates with each of the toxin subtypes Stx1+Stx2a, Stx1+Stx2c, Stx2a, and Stx2c were identified (Table15, Figure 25). The percentage of isolates with Stx1+Stx2a decreased from 75.0% in 1996 to 45.0% in 2005. There were no significant trends in the percentage of isolates with Stx1+Stx2c or Stx2a between 1996 and 2005. The percentage of isolates with Stx2c increased from 4.2% to a high of 26.7% in 2005. Therefore, the decrease in the frequency of isolates with Stx1+Stx2 is almost exclusively a decrease in Stx1+Stx2a, whereas the increase in Stx2-only is mainly caused by an increase in Stx2c.

Table 15: Stx2 Toxin Subtypes of STEC O157 Isolates (na: no amplification), 1996-2005 [NARMS Dataset, (n=363)]

	Number of Isolates in Dataset	Stx1+Stx2a	Stx1+Stx2c	Stx2a	Stx2c	na
1996	24	18 (75.0%)	1 (4.2%)	4 (16.7%)	1 (4.2%)	0 (0.0%)
1997	23	13 (56.5%)	0 (0.0%)	8 (34.8%)	2 (8.7%)	0 (0.0%)
1998	26	19 (73.1%)	0 (0.0%)	2 (7.7%)	3 (11.5%)	2 (7.7%)
1999	27	18 (66.7%)	1 (3.7%)	6 (22.2%)	2 (7.4%)	0 (0.0%)
2000	28	12 (42.9%)	2 (7.1%)	11 (39.3%)	1 (3.6%)	2 (7.1%)
2001	28	14 (50.0%)	1 (3.6%)	7 (25.0%)	5 (17.9%)	1 (3.6%)
2002	28	14 (50.0%)	1 (3.6%)	4 (14.3%)	6 (21.4%)	3 (10.7%)
2003	58	27 (46.6%)	3 (5.2%)	13 (22.4%)	8 (13.8%)	7 (12.1%)
2004	61	26 (42.6%)	3 (4.9%)	7 (11.5%)	14 (23.0%)	11 (18.0%)
2005	60	27 (45.0%)	3 (5.0%)	11 (18.3%)	16 (26.7%)	3 (5.0%)
Total	**363**	**188**	**15**	**73**	**58**	**29**

Figure 25: Stx2 Toxin Subtypes of STEC O157 Isolates (percentage of isolates) (na: no amplification), 1996-2005 [NARMS Dataset, (n=363)]



CHAPTER V: DISCUSSION

Study Significance

STEC O157:H7 is responsible for causing approximately 73,000 illnesses and 61 deaths annually in the United States (Mead, 1999). All STEC produce one or both of two Shiga toxins, Stx1 and Stx2. Findings from previous studies indicate that STEC strains that produce Stx2 are more strongly associated with HUS than strains that produce both Stx1 and Stx2 or only Stx1. In recent years, CDC epidemiologists seem to have noticed a recent increase in the rate of HUS among STEC outbreaks. Such an increase could be due to a shift in the toxin type produced by STEC strains. In an effort to identify if such a shift exists, this study compares the toxin profiles of human STEC O157 strains within three independent datasets, collected by PulseNet, eFORS, and NARMS. The trends in HUS rates reported through eFORS were also studied.

Important Study Findings

It was hypothesized that 1) there has been an increase in the number of foodborne outbreaks caused by STEC O157:H7 during the time period 1999-2008 and 2) there has been an increase in the number of STEC O157:H7 strains that produce Shiga toxin 2-only during the time period of 1999-2008. The study findings discussed below illustrate that the first hypothesis is probably not true whereas the latter may be true according to the data analyzed.

Results from the PulseNet dataset show that since 2002, the number of foodborne outbreaks caused by STEC O157:H7 has increased, whereas the number of outbreaks

reported through eFORS did not show any trends from 1998-2006, with an annual average reported number of 25.8 outbreaks. Fewer outbreaks were reported in PulseNet in 2002-2004 than in eFORS, whereas the number of outbreaks reported in PulseNet was higher than in eFORS for the years 2005-2006. This indicates that significant under-reporting of outbreaks took place in PulseNet until 2004. It is to be expected that the number of outbreaks detected in PulseNet is higher than in eFORS, since the eFORS database only contains confirmed outbreaks whereas the PulseNet database also includes clusters that are not investigated epidemiologically. A reason for the under-reporting in PulseNet in 2002-2004 may be that during the early years following introduction of cluster codes, clusters were predominantly assigned cluster codes if they were investigated by epidemiologists. Since 2004, all clusters detected by PulseNet have been assigned a cluster code.

In the PulseNet dataset, the number of outbreaks was lowest in 2003 at 8 outbreaks and highest in 2005 at 54 outbreaks. However, after 2005, the number of outbreaks gradually decreased to a low of 39 outbreaks in 2008. The average number of outbreaks per year as seen in the PulseNet dataset was 33.1. The increase in the number of outbreaks per year in the PulseNet dataset may be artificial for the reason explained above and perhaps because of improved cluster detection and expansion of the PulseNet network. The number of outbreaks for 1999-2001 was not available, as PulseNet did not begin using outbreak codes until 2002.

In contrast to the results found using the PulseNet dataset, there was not a gradual increase in the number of outbreaks reported to eFORS during the time period 1998-2006 (data for 2007 and 2008 were not available). The number of outbreaks reported to eFORS

ranged from a low of 17 outbreaks in 2004 to a high of 33 outbreaks in 2006. The average number of outbreaks per year as seen in the eFORS dataset was 25.8.

Results from this study also show that since 1999, the number of STEC O157:H7 strains that produce Shiga toxin 2-only has increased. The PulseNet dataset of 4,402 isolates showed a gradual increase in the number of strains producing Stx2-only from 1999 to 2008. The percentage of isolates producing Stx2-only increased from a low of 11.5% in 1999 to a high of 57.2% in 2008. This increase was found to be statistically significant ($p < 0.0001$). This increase occurred in parallel to a gradual decrease in the percentage of isolates producing Stx1+Stx2.

When isolates within the PulseNet dataset were separated into outbreak isolates and sporadic isolates, the same trend was seen. Among the 1,629 outbreak isolates, the percentage of isolates producing Stx2-only increased from a low of 31.0% in 2002 to a high of 59.4% in 2008. Among the 2,519 sporadic isolates, the percentage of isolates producing Stx2-only increased from a low of 21.5% in 2002 to a high of 55.8% in 2008. These increases were found to be statistically significant ($p<0.0001$).

Results from the eFORS dataset also showed an increase in the number of outbreaks producing Stx2-only relative to the other toxin types for the time period 2000-2006 (Figure 22). No toxin information was available for any of the outbreaks prior to 2000. During 2000-2004, there were six outbreaks in which the toxin information was known, and none of these outbreaks were caused by Stx2-only producing strains. In 2005, there were 17 outbreaks with known toxin information, of which 10 (58.8%) were caused by Stx2-only producing strains. In 2006, there were 16 outbreaks with known toxin information, of which 11 (69.0%) were caused by Stx2-producing strains.

Additionally, results from the NARMS dataset of 363 isolates showed a gradual increase in the number of isolates producing Stx2-only during the time period 1996-2005. The percentage of isolates producing Stx2-only increased from 20.8% in 1996 to 45.0% in 2005. This increase occurred in parallel to a gradual decrease in the percentage of isolates producing Stx1+Stx2. Isolates producing Stx1-only remained at a steady low for all years. This trend was found to be statistically significant ($p<0.0101$).

Further analysis of the NARMS dataset revealed trends in the Stx2 toxin subtypes of the isolates. Results in this dataset showed that the increase in Stx2-only producing strains is mainly caused by an increase in Stx2c. The percentage of isolates with Stx2c increased from 4.2% in 1996 to 26.7% in 2005. This increase occurred in parallel to a gradual decrease in the percentage of isolates producing Stx1+Stx2a, indicating that the decrease in frequency of isolates with Stx1+Stx2 is almost exclusively a decrease in Stx1+Stx2a.

It was hypothesized that there would be observable trends in the demographic characteristics (including age and gender) of isolates belonging to each toxin profile for the time period 1999 to 2008. However, findings from this study show that in humans, there is no correlation between age and toxin profile of the infecting STEC O157 strain. Among the 3,714 isolates with a known patient age in the PulseNet dataset, there were no differences in the median age when the median ages were compared by year, but when the median age of all isolates were compared for all years combined, the median age of all isolates with Stx1+Stx2 was different from the median age of all isolates with Stx2-only, and this difference was found to be statistically significant ($p<0.0001$). The median

ages of patients were compared instead of the mean ages, since the data in the PulseNet dataset is not normally distributed.

Findings from previous studies specify young age as a risk factor for STEC O157 infection, (Scheiring 2008), which was also found in this study. When the 3,714 isolates in the PulseNet dataset with toxin and age information were examined by age interval, the age intervals with the highest numbers of cases were 1-5 years, 6-10 years, and 11-15 years, for each toxin type. Results from this study show that the prevalence of STEC O157:H7 is a major public health concern because contaminated products may be consumed by patients of all ages but that young age is a risk factor.

This study also shows a slightly higher risk of infection among females when toxin profiles were compared between the two genders. In the 3,639 human STEC O157 isolates with known gender information, the average percentage of female cases for all isolates with Stx1-only, Stx1+Stx2, and Stx2-only was 57.9%, 53.7%, and 54.0%, respectively. Thus, the percentage of female cases was slightly above 50% for each of the toxin types. This finding is consistent with results of previous studies that specify female gender as a risk factor for STEC O157 infections (Scheiring 2008), however, the difference was not found to be statistically significant in this study (p=0.243).

As hypothesized, this study showed an observable seasonal trend in STEC O157 infections during the time period 1999-2008. Findings showed a general seasonal increase between the second quarter (April-June) and third quarter (July-September) for each toxin type within every year, and a general seasonal decrease between the third quarter and fourth quarter (October-December) for each toxin type within every year. The number of isolates was consistently lowest during the first quarter (January-March) for

each toxin type and year. These data are consistent with results of previous studies that indicate a marked summer peak in prevalence of STEC O157:H7 (Crump 2003). This seasonality is unexplained, but is also observed for other bacterial enteric infections and may be due to ecological factors or increased exposures (ex: recreational water, undercooked foods served at barbecues, etc.) during warmer summer months.

Results from this study did not illustrate any trends in the geographical distribution of toxin types. When all isolates from 1999-2008 were combined for each toxin profile, results showed the highest concentration of cases in California, Michigan, Ohio, and Virginia, regardless of toxin profile. When isolates of each toxin profile were compared in 1999 versus 2008, the same states had the highest concentrations of cases. Higher population densities, agricultural, ranching, and beef processing activities, and/or the presence of "supershedders" (cattle with greater *E. coli* O157:H7 transmission potential) may be contributing factors to the higher concentration of cases in these states. In addition, Michigan and Virginia serve as PulseNet Area Laboratories. In this role they provide surge capacity for surrounding states, which may attribute to a higher number of cases being submitted by these two states. Thus, the observed geographical distribution could possible be explained by differences in the public health laboratories' capacity to perform Stx-type determination.

In this study, PFGE data showed that certain toxin types were associated with particular PFGE patterns. This finding was based on the top ten PFGE patterns in the *E. coli* national database. One hundred percent of isolates with PFGE pattern EXHX01.1486 produced Stx2-only. For patterns EXHX01.0047, EXHX01.0200, EXHX01.0224, EXHX01.0124, EXHX01.0125, there was a strong association between the toxin profile

and PFGE pattern, as more than 90% of isolates with these patterns produced Stx2-only. For patterns EXHX01.0074, EXHX01.1343, EXHX01.0087, and EXHX01.0008, more than 80% of the isolates produced Stx1+Stx2. None of the patterns among the top 10 produced Stx1-only. This association between toxin type and PFGE pattern is noteworthy because it suggests that it may be possible to predict the toxin type associated with an outbreak based on the PFGE pattern of the isolates belonging to the outbreak.

Among the top five PFGE patterns with Stx1-only, there were only two patterns (EXHX01.3417 and EXHX01.3138) that were exclusively seen with Stx1-only when compared against the entire *E. coli* national database; however each of these two patterns had only been seen twice in the entire database, therefore there was not a large enough sample to conclude that these patterns are always associated with Stx1-only producing strains.

It was hypothesized that non-O157 strains would be associated with toxin type Stx1-only. This hypothesis was found to be true in general, as analysis of the distribution of toxin types among the top six non-O157 STEC serotypes showed that the percentage of isolates with Stx1-only was higher than the percentage of isolates with Stx1+Stx2 or Stx2-only. However, upon a closer look, serotype-specific differences were noticed. In *E. coli* O121 strains, the percentage of isolates with Stx2-only was significantly higher than the other toxin types. Strains of serotype O111 were almost evenly distributed between Stx1-only and Stx1+Stx2, and all three toxin profiles were almost evenly distributed in serotype O145. All serotypes contained strains with each toxin profile. These findings illustrate that outbreaks caused by *E. coli* O121 may be more severe than outbreaks caused by other non-O157 STEC serotypes due to the presence of the Stx2 toxin.

Additionally, the serotype alone should not be used in predicting the risk of severe illness caused by an STEC strain. As previous studies have indicated, the combined presence of the eae and Stx2 genes are essential in predicting the severity of illness, rather than the serotype.

Results from this study do not indicate any trends in HUS rates among STEC O157 outbreaks in the United States from the time period 1998-2006. HUS rates were known for 166 (71.2%) of the 233 outbreaks in the eFORS dataset and the average HUS rate for all outbreaks ranged from 0.40% in 1998 to 16.1% in 2006. However, many of the HUS rates in the eFORS dataset were much higher than would be expected (as high as 50.0%), based on outbreaks investigated by CDC epidemiologists and information in the literature, therefore these data may be unreliable for identifying trends in HUS rates. Some reported outbreaks seemed to have been caused by strains containing Stx1-only. However, in many outbreaks, including one out of three Stx1-only producing outbreaks, the toxin profile was only determined from one strain. Shiga toxins are encoded on mobile genetic elements, called phages, that may be lost. The toxin profile of an outbreak strain should therefore be determined for several outbreak-related isolates.

An explanation for the unusually high HUS rates seen in the eFORS dataset may be a systematic reporting error or bias toward preferentially reporting severe illness. Taking into account that the HUS rates reported to eFORS may be unreliable, no correlations between toxin profiles and HUS rates were made using this dataset.

Study Limitations

It is important to discuss the limitations involved in this study. One limitation is that the PulseNet dataset is that *E. coli* national database mirrors the surveillance performed in the states, and sampling and reporting of STEC O157 isolates varies from state to state. Furthermore, the toxin type and demographic information is not submitted for every isolate uploaded to the PulseNet database, and therefore this study excludes isolates where this information was not available. This is a limitation because the dataset does not include every STEC O157 isolate submitted to the *E. coli* national database between 1999 and 2008. However, since over 70 U.S. public health laboratories and federal regulatory agencies regularly submit isolates to the PulseNet database, this study assumes isolates included in the PulseNet dataset represent the national trend of infection (Gerner-Smidt 2006). Another limitation of the PulseNet dataset is that PulseNet data is biased toward outbreak isolates; therefore, the *E. coli* national database may not contain an accurate representation of trends among sporadic isolates. However, the trends seen in the PulseNet dataset were confirmed by the random sampling of the NARMS dataset.

A limitation of the eFORS dataset is that only 71.2% of STEC outbreaks in the dataset contained HUS rates, and HUS information was not provided for all patients in every outbreak. Additionally, only a small percentage (25.9%) of these outbreaks could be linked to outbreaks in the PulseNet dataset with toxin information. Because HUS is a nationally notifiable disease, eFORS is biased toward HUS cases (CDC-DISSS 2009). This also showed up in many of the outbreaks in the eFORS dataset, where the HUS rates were unrealistically high and not representative of typical STEC O157 outbreaks.

A limitation of the NARMS dataset is that a small percentage of the isolates included in the dataset revealed no amplification following PCR testing. These isolates were repeated with a different PCR assay, which differed from the original assay by targeting slightly different regions of the Stx genes. Some isolates still revealed no amplification, which may have been due to mutations in the toxin genes, or the organism being non-toxigenic O157, which would have led to the toxin genes being absent or too broken to be amplified. However, since the number of non-amplifying isolates was low, this limitation would have little effect on the conclusions from this part of the study.

Another limitation of the study was that the three datasets all contained isolates from slightly different time periods; the PulseNet dataset contained isolates from 1999-2008, the eFORS dataset contained isolates from 1998-2006, and the NARMS dataset contained isolates from 1996-2005. The time frames for the PulseNet and eFORS dataset were defined to represent the most comprehensive information that was available in each dataset, while the time frame for the NARMS dataset was defined by a randomized sampling scheme of available isolates. The sample sizes of each dataset also differed for each dataset which may also be a limitation to this study.

Recommendations and Future Studies

It is recommended to compare toxin profiles of STEC O157 strains to HUS rates from a different data source. The data source used in this study for capturing HUS rates was biased toward HUS cases, leading to unreliably high HUS rates among STEC O157 outbreaks. Additionally, in this study, outbreaks in one dataset (eFORS) had to be linked with outbreaks in another dataset (PulseNet) to obtain the toxin profiles of those

outbreaks. It is recommended that state and public health laboratories submit as much information as possible to each of these data sources, allowing the data to be linked more efficiently.

Future studies could investigate trends in toxin profiles among non-human isolates; i.e. food sources and animal reservoirs. This study revealed an increase in the number of strains producing Stx2-only relative to strains producing Stx1-only and Stx1+Stx2, therefore it would be reasonable to assume that the same trend exists in the food commodities and animal reservoirs that are responsible for causing illness in humans. The data obtained from such a study could be used to understand if certain food commodities are associated with certain toxin profiles which may cause more severe illness in humans. Additionally, future studies could examine if the shift in toxin profiles seen in this study also exists in other countries. Because of the global nature of today's food supply, it is likely that these trends are mirrored in other parts of the world.

CHAPTER VI: CONCLUSIONS

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is a major cause of foodborne illness in the United States and causes severe gastroenteritis and may cause life-threatening HUS, the most serious complication of STEC infection. One of the main virulence factors of STEC infections is the production of one or more type of *Shiga* toxin (Stx1, Stx2, or both).

The first aim of this study was to determine if data collected from two independent datasets showed an increase in the number of outbreaks caused by STEC O157:H7 during the time period 1999-2008. Using the PulseNet *E. coli* national database and outbreaks reported to eFORS, the number of outbreaks occurring within each year was determined based on PulseNet-assigned outbreak codes. The overall conclusion is that there were no observable trends in the number of outbreaks occurring since 2002.

The second aim of this study was to determine if data collected from three independent datasets showed an increase in the number of STEC O157:H7 strains that produce Shiga toxin 2-only. The PulseNet dataset showed a gradual increase in the number of strains producing Stx2-only from 1999 to 2008, in parallel to a gradual decrease in the percentage of isolates producing Stx1+Stx2. The same trend was seen when outbreak-related isolates were separated from sporadic isolates. The eFORS and NARMS datasets also showed an increase in the number of outbreaks producing Stx2-only relative to the other toxin types for the time period observed. Further analysis of the NARMS dataset revealed that the increase in Stx2-only producing strains is mainly

caused by an increase in Stx2c, relative to Stx2a. Thus, a shift in the toxin profiles has changed during the study period.

The third aim of this study was to determine if data collected from eFORS reports showed an increase in the HUS rates of STEC O157:H7 outbreaks during the time period observed. However, no conclusions could be made regarding trends in HUS rates based on the data available in the eFORS dataset since this surveillance system currently seems to be unreliable for HUS surveillance.

Finally, this study attempted to identify any other observable trends among toxin profiles of STEC O157 strains. Demographic characteristics including patient age and gender were examined, in addition to geographical and seasonal trends. Results showed a higher number of cases among children (1-15 years old) and a slightly higher incidence of infection among females, regardless of toxin type. Seasonal trends were identified, as there was a general increase in cases between the second and third quarters of each year, for each of the three toxin types and a general decrease in cases between the third and fourth quarters of each year for each toxin type. Analysis of geographical trends revealed a higher concentration of cases in California, Michigan, Ohio, and Virginia, relative to other states, for all three toxin profiles, when the years 1999-2008 were combined. However, the reliability of this finding may be questioned.

Overall, this analysis shows a dramatic shift over time in the toxin profiles of human STEC O157 strains in the United States towards strains that produce only Stx2. This shift was observed in three independent databases of mostly sporadic isolates and in outbreak isolates. No conclusions could be made regarding trends in HUS rates. A systematic and reliable reporting method of HUS cases among sporadic and outbreak

associated STEC infections in the United States is warranted in order to understand if a correlation exists between the severity of this disease and the virulence of the infecting strain. Further work is needed to determine if a similar trend in toxin profiles of STEC O157 strains has occurred in animal reservoirs, foods, and other countries. There is a great need to recognize the trends in toxin profiles of STEC O157 strains in order to fully understand their potential for causing human illness.

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