EPIDEMIOLOGY OF SHIGA TOXIN-PRODUCING *E. COLI* (STEC) IN THE FINISHING PIGS AND HUMANS

By

Marion Tseng

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Comparative Medicine and Integrative Biology-Doctor of Philosophy

2014

ABSTRACT

EPIDEMIOLOGY OF THE SHIGA TOXIN-PRODUCING *E. COLI* (STEC) IN THE FINISHING PIGS AND HUMANS

By

Marion Tseng

Shiga toxin-producing *E. coli* (STEC) infections are a significant public health concern. The epidemiology of STEC in swine remains largely unknown, and the role of swine play in STEC transmission to humans is not yet elucidated. The objectives of this dissertation are to provide descriptive epidemiology of STEC shedding in the finishing pigs, characterizing swine STEC using molecular methods, and to understand the epidemiology of STEC, specifically STEC in non-O157 serotypes, in human STEC cases in Michigan.

A descriptive longitudinal study was conducted to investigate the fecal shedding of STEC in the finishing pigs. Three cohorts of finishing swine (n=50/cohort; total 150 pigs) were included in the longitudinal study. Individual fecal samples were collected every 2 weeks (8 collections/pig) from the beginning (pig age=10 weeks old) to the end (pig age =24 weeks old) of the finishing period. STEC isolates were recovered in at least one sample from 65.3% (98/150) of the pigs, and the frequency distribution of first-time STEC detection during the finishing period resembled an outbreak curve. Nineteen O:H serotypes were identified among the STEC isolates. Most STEC isolates (n=148) belonged to serotype O59:H21 and carried the stx_{2e} gene. One O49:H21 STEC isolate carried the stx_{2e} and *eae* gene. High prevalence rates of STEC during the finishing period were observed, and STEC isolates in various non-O157 serotypes were recovered.

To investigate whether there were actual STEC outbreaks within the finishing pigs as suggested by the descriptive epidemiologic study, a subset of swine O59:H21 STEC strains

(n=29) was analyzed by pulsed field gel electrophoresis (PFGE) to examine their genetic relatedness. Moreover, the presence and absence of a large panel of virulence genes was examined in a subset of swine STEC strains (n=155) recovered by a high-throughput real-time PCR array system. Seventeen different combinations of virulence gene profiles and serotypes were determined in the swine STEC strains. The majority of the swine O59:H21 STEC strains (n=120) carried the same virulence gene profile. Genes encoding adhesins were identified, for example, the *iha* gene (n=154). The PFGE results revealed that swine STEC strains from pigs raised in the same finishing barn were closely related, supporting the observations that there were STEC outbreaks within the finishing barn. This work is the critical first step to understand the swine STEC epidemiology and potential pathogenic mechanisms of swine STEC in human disease.

To better understand the demographic and clinical characteristics of STEC cases, specifically non-O157 STEC, STEC cases reported to the Michigan Department of Community Health (MDCH) from 2001 through 2012 were described. An increasing trend of non-O157 STEC cases was observed in this 12-year period, and the incidence rates were similar for O157 and non-O157 STEC cases in 2012. No demographic characteristics were significantly different between O157 and non-O157 STEC cases. However, the odds of hospitalization were 2.36 times higher in O157 STEC cases than in non-O157 STEC cases when adjusted for age and gender. The information enhances our understanding in epidemiology of non-O157 STEC in Michigan, and future research is warranted to understand these pathogens in order to improve prevention and control efforts.

ACKNOWLEDGEMENTS

I would like to express my gratitude to all of the people who have contributed to this PhD dissertation research. This dissertation represents the accumulated efforts by all the collaborators and people who have made contributions. I am forever grateful for all of the assistance and support throughout my graduate school career, and without these helps I will not be able to complete this dissertation.

First and the most importantly, I would like to acknowledge my major advisor, Dr. Julie Funk. It is an honor and it has been a true pleasure to work under Dr. Funk's guidance, support, and encouragement. Words cannot describe how grateful I am to have Dr. Funk as my PhD advisor. Dr. Funk has not only enlightened me as to scientific research, but also inspires me to become a better person.

I would like to thank my PhD guidance committee: Dr. Paul Bartlett, Dr. Shannon Manning, and Dr. Melinda Wilkins. It has been very rewarding and pleasant to work with each one of them, whether it is for scientific research or guidance for my future career goal.

I sincerely thank all the collaborators of my research projects. First of all, I would like to thank Dr. Pina Fratamico at the USDA ARS ERRC (U.S. Department of Agriculture, Agricultural Research Services, Eastern Regional Research Center) for all of the efforts contributing to this research project. Dr. Fratamico has provided priceless inputs and support into the microbiology component of this research, and it has been a pleasure to collaborate with her. I also would like to thank other members at the USDA ARS ERRC, Lori Bagi and Danielle Manzinger, for their efforts and assistance contributing to sample processing and microbiologic experiments. I would like to acknowledge Dr. Patrick Fach and Dr. Sabine Delannoy at the

ANSES (French Agency for Food, Environmental and Occupational Health and Safety) for their efforts and collaboration for determining the virulence gene profiles for the bacterial strains.

I sincerely thank all the following people who have provided assistance in part of my dissertation research: Dr. Chitrita Debroy at *E. coli* Reference Center, the Pennsylvania State University for the STEC strains characterization, MSU DCPAH (Diagnostic Center for Population and Animal Health) Bacteriology, specifically Niesa Kettler and Terrence MacManus for their assistance in molecular genotyping methods for swine STEC, and the Michigan Department of Community Health for their assistance in the data collection of human STEC cases.

I also would like to thank the swine producers who have participated in this research project. Many thanks to the undergraduate and veterinary students at MSU who helped with the visits to the swine farms, sample collections, and assisting with the data management: Alexandra Buckley, Allan Mergener, Tasha Likavek, and Joel Sparks.

I would like to acknowledge my former colleague Dr. Alda Pires for all the assistance with swine farm visits, sample collections, data management, and statistical analysis. It has been wonderful to have Dr. Pires as a colleague and friend throughout the graduate school. Many thanks to Dr. Pires' support and all the good memories on and off work.

I also would like to thank Dr. Vilma Yuzbasiyan-Gurkan for all the assistance and support as a graduate program director through my graduate school. I also would like to acknowledge my friends at MSU for their support, assistance and friendship. Finally, but not the least, I am grateful for the support from my family and friends. Specifically, I truly appreciate my parents, who always support and encourage me to pursue my professional career. Most importantly, I am forever grateful for the support from my husband, who has been always there

for me, including in-house scientific discussion involving *E. coli*. I would like to thank for the support from the U.S. Department of Agriculture, National Needs Fellowship in Epidemiology and Infectious Disease, National Pork Board (grant number 12-069), the U.S. Department of Agriculture, National Institute of Food and Agriculture, Agriculture and Food Research Initiative (award number 2013-67005-21189), and Michigan State University the Graduate School Dissertation Completion Fellowship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

EHEC Enterohemorrhagic *E. coli*

HC Hemorrhagic Colitis

HUS Hemolytic Uremic Syndrome

MLVA Multilocus Variable Number Tandem Repeat Analysis

PCR Polymerase Chain Reaction

PFGE Pulsed-Field Gel Electrophoresis

STEC Shiga toxin-producing *E. coli*

CHAPTER 1

Literature review: Shiga toxin-producing *Escherichia coli* in swine

Manuscript based on the contents of this chapter has been published in M. Tseng, P. M. Fratamico, S. D. Manning, J. A. Funk · 2014. Shiga toxin-producing *Escherichia coli* in swine: the public health perspective. Animal Health Research Reviews. doi:10.1017/S1466252313000170. (Published on Animal Health Research Reviews, first view on January 8th, 2014)

ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) strains are food-borne pathogens that are an important public health concern. STEC infection is associated with severe clinical diseases in human beings, including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), which can lead to kidney failure and death. Cattle are the most important STEC reservoir. However, a number of STEC outbreaks and HUS cases have been attributed to pork products. In swine, STEC strains are known to be associated with edema disease. Nevertheless, the relationship between STEC of swine origin and human illness has yet to be determined. This review critically summarizes epidemiologic and biological studies of swine STEC. Several epidemiologic studies conducted in multiple regions of the world have demonstrated that domestic swine can carry and shed STEC. Moreover, animal studies have demonstrated that swine are susceptible to STEC O157:H7 infection and can shed the bacterium for two months. A limited number of molecular epidemiologic studies, however, have provided conflicting evidence regarding the relationship between swine STEC and human illness. The role that swine play in STEC transmission to people and the contribution to human disease frequency requires further evaluation.

INTRODUCTION

Infection with Shiga toxin-producing *Escherichia coli* (STEC) is a critical public health concern. STEC infections are associated with outbreaks and sporadic cases of diarrhea and severe clinical diseases in human beings, including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (1, 2). HUS is a life-threatening thrombotic disorder characterized by acute renal failure, thrombocytopenia and microangiopathic hemolytic anemia (1, 3, 4). It is one of the leading causes of acute renal failure in young children worldwide (4-6). Every year in the U.S., more than 170,000 human illnesses are attributed to STEC (7), and food-borne STEC illnesses represent an estimated economic burden of 280 million dollars (8).

STEC represent a subset of *E. coli* that produce a cytotoxin known as the Shiga toxin (Stx), or verotoxin. Various STEC transmission routes have been identified, though STEC infections are most frequently associated with consumption of contaminated food (meat, dairy products, produce, and others) and water (9-11). Other sources of infection include animal contact (12-14), person-to-person contact (15-17), and airborne transmission (18). Cattle have been suggested to be the important animal reservoir of STEC, and they do not typically present with any STEC-associated clinical symptoms (9, 19, 20), except in calves under experimental conditions (21). Nevertheless, many other food products, including pork products, have been confirmed as vehicles of STEC transmission (22-28). Feral swine, for example, were found to be a risk factor for STEC O157:H7 contamination of the spinach implicated in a 2006 North American outbreak (29). In pork-associated outbreaks and cases, it is unknown whether the contamination of pork products occurs during swine processing or via cross-contamination from other foodstuffs (25, 27, 28, 30). Although swine-associated outbreaks have been reported less

frequently than cattle-associated outbreaks, the likelihood that swine represent an important source of STEC infections in human beings cannot be overlooked.

The role that swine play in STEC transmission to human beings needs to be further evaluated to determine whether swine-derived STEC strains are an important public health concern. The severe symptoms associated with STEC infections and the increasing frequency of infections caused by a large variety of STEC serotypes highlight the fact that more research is needed to better understand these pathogens to aid in developing prevention and control strategies. In this literature review, previous studies focusing on STEC of swine origin will be critically summarized.

FOOD-BORNE OUTBREAKS ASSOCIATED WITH PORK PRODUCTS

Though only in a few instances, pork has been reported as a potential vehicle involved in outbreaks of STEC infections (22-28). Table 1.1 summarizes the food products involved in these outbreaks and the serotype and virulence gene characteristics of the STEC strains isolated in these outbreaks. Mixed meat products were involved in most of these reported outbreaks. Thus, it was difficult to attribute the source of contamination to any of the animal species. Notably, the most recent outbreaks were associated with salami containing only pork (27) and large cuts of pork from a whole roasted pig (28). It is impossible to eliminate the possibility that cross-contamination from different foodstuffs took place during processing. However, pigs were suggested to be the source of STEC O157:H7 infection in the most recent outbreak (28). Though the responsible source of STEC was not conclusive in these outbreaks, pork products cannot be excluded as a potential vehicle of STEC transmission.

STEC PREVALENCE IN SWINE POPULATIONS

Although outbreaks have been rarely reported, the incidence of human STEC outbreaks associated with pork products has raised questions about the sources of STEC in the food chain, from pork products, pigs at slaughter facilities, to the most upstream origin, on-farm swine. The presence of STEC in pork or pork-associated products has been reported in some epidemiologic studies (31-37). These data were sparse, and the numbers related to STEC prevalence in pork or pork-associated products were not consistent. The discrepancies are potentially attributable to multiple factors. For example, different isolation protocols and different types of pork products were used in these studies. In short, these positive findings of STEC in pork and pork-associated products indicate that STEC are present on retail pork products. The possibility that swine play a role in the contamination of pork products cannot be excluded.

The prevalence of STEC in clinically healthy swine populations has been reported in numerous studies in multiple regions of the world. In the U.S, STEC detection in the swine population has been reported. A study was published in which colon samples were obtained from pigs at a U.S. slaughter facility to detect the presence of STEC O157 (38). Six (1.9%) of the 305 colon samples were positive for STEC O157:H7. Interestingly, no STEC O157:H7 isolate was recovered in the National Animal Health Monitoring System's (NAHMS) Swine 1995 study (39) and the later NAHMS 2000 study (38). However, it is impossible to directly compare the number of STEC O157:H7 positive samples in these studies due to different study designs and sample collection methods. In short, these reports provided evidence that swine in the U.S. carry STEC serotype O157:H7, although at a much lower rate compared to cattle.

Besides STEC O157:H7, the NAHMS 2000 study also recovered and characterized non-O157 STEC isolates (40, 41). At least one STEC isolate was recovered from 196 (28.5%) of 687 fecal samples. A total of 219 STEC isolates were recovered, and they were categorized into various non-O157 O serogroups. Most importantly, some O serogroups had previously been associated with human clinical cases, namely O9, O20, O91, O101, and O121. The results of this study indicated that clinically healthy pigs from multiple states in the U.S. shed non-O157 STEC at some point during the late finishing period.

A recent study reported no significant effect of antimicrobials (chlorotetracycline and bacitracin) in feed on fecal shedding of non-O157 STEC in finishing swine (42). Notably, STEC belonging to important O serogroups, namely O26, O103 and O145, were isolated from 6.9, 2.4, and 4.8% of all the swine rectal swab samples. However, this study targeted a selected group of non-O157 STEC for detection in the samples (O26, O103, O111, O121, O145). In spite of that, the high prevalence of the Shiga toxin gene (*stx*) in enriched samples (58% prior to slaughter) and the detection of non-O157 STEC belonging to important O serogroups highlight the need to further investigate STEC shed by clinically-healthy swine.

Besides the U.S., studies in other regions of the world reported wide ranging estimates of STEC prevalence from 0% (43, 44) to 68.3% (45). The highest prevalence, 68.3%, was reported from one study in Chile (45). Rectal swab samples were collected during the evisceration process from swine and cattle at slaughter facilities. Surprisingly, the STEC prevalence in swine samples was higher than that in cattle (28.7%). In addition to the strains identified as non-typable by O serogrouping, O157 was the most prevalent O serogroup among the swine STEC isolates. Other important STEC serogroups, such as O26 and O111, have also been reported (46). In one state in Brazil, the prevalence of STEC was 1.4% (one STEC-positive sample) from

74 fecal samples obtained aseptically from swine intestines at slaughter facilities (47). A later study performed in another state in Brazil reported one (0.4%) STEC O103 isolate recovered from 215 sponge-rubbed samples from swine carcasses at three slaughter facilities (48).

In a South African study, *E. coli* O157:H7 was more frequently found in swine fecal samples and in pork (67.7%) than in cattle fecal samples and beef (27.7%) (36). In this study, only a selected subset of *E. coli* O157:H7 isolates was analyzed for the presence of *stx*. Therefore, it is difficult to determine the number of STEC O157:H7 positive samples in this study. Nevertheless, this study was in agreement with the previous Chilean studies (45, 46) showing that swine are able to shed STEC at a relatively high rate and are potentially important STEC reservoirs in some places in the world.

Several European studies have reported STEC prevalence in swine populations, and most of the earlier studies focused on STEC serogroup O157. A study conducted in the United Kingdom reported the recovery of six (0.3%) STEC O157 isolates from over 2000 swine fecal samples at slaughter facilities (49). Similarly, in Ireland, the prevalence of STEC O157:H7 was 0.6% (3 of 480) in fecal samples of pigs at slaughter facilities (50). In a study conducted in the Netherlands, STEC O157 was isolated from 1 (0.7%) of 145 fecal samples from pigs at one slaughter facility (51). A low proportion (0.7%, 1 of 150) of STEC O157-positive fecal samples was reported from pigs at slaughter facilities in Northern Italy (52). In Norway, STEC O157:H7 was found in 2 (0.1%) samples of intestinal contents from 1976 pigs, and one of the two pigs was housed on a farm that also reared cattle. However, no STEC O157:H7 isolate was recovered from samples of cattle on the same farm (53). Interestingly, other studies also reported isolation of STEC O157:H7 strains from pigs that were kept with cattle or other ruminants on the same farm. For instance, STEC O157:H7 was recovered from 2 (0.08%) of 2446 fecal samples of

slaughtered pigs that were housed with ruminants on the same farm in a Swedish study (54). In general, the prevalence of STEC O157:H7 in swine appeared to be low in these studies. However, the presence of ruminants on the same farm that reared swine suggests that horizontal transmission of STEC may occur between different animal species.

Several studies investigated the prevalence of both STEC O157 and non-O157 in clinically healthy swine populations. A study in Belgium reported that non-O157 STEC strains were isolated from 8 (7.9%) out of 101 swine colon fecal samples at a slaughter facility (55). In Switzerland, the *stx* gene was detected in 138 (22%) of the 630 swine fecal samples at a slaughter facility. Subsequently, forty-five non-O157 STEC isolates were recovered from 45 randomly-selected *stx*-gene-positive samples (56). It was difficult to estimate the true prevalence of STEC in this study because not every *stx*-positive sample was analyzed for STEC isolates. These reports suggest that swine may be potential reservoirs of non-O157 STEC. In these studies, relatively low numbers of STEC isolates were recovered even though the numbers of *stx*-positive samples were high. This fact reflects the challenges of non-O157 STEC isolation. In contrast to the above positive findings, one study conducted in Northern Spain analyzed pooled rectal fecal samples from a total of 510 pigs, and the test results were negative for both O157 and non-O157 STEC (43). Negative isolation of STEC in 106 swine fecal samples was also reported by a study conducted in Central Greece (44).

The presence of STEC in swine populations has also been documented in Asia. In Japan, the prevalence of STEC O157:H7 in clinically healthy on-farm swine was 1.4%, and this number was similar to the prevalence in Japanese cattle around the same time period (57). A later Japanese national surveillance report stated that STEC isolates were recovered from 32 (14%) of 179 swine fecal samples. Some strains belonged to the serotypes frequently associated with

human diseases (58). In Korea, only reports of STEC O157:H7 in swine have been published. One (0.3%) STEC O157:H7 isolate was recovered from 345 fecal samples from pigs at slaughter facilities and on farms (33). Two STEC O157:H7 isolates were recovered from fecal samples of pigs at slaughter facilities in another study (59). However, the prevalence was unknown because the total number of samples from pigs was not provided. In a recent report from Korea, one (0.3%) STEC O157 isolate was recovered from 291 swine fecal samples (60). In China, one study reported STEC isolates recovered from 10 (2.1%) of 487 rectal swabs from pigs at a slaughter facility in Hong Kong. The pigs were from a number of provinces in mainland China (61). Another study reported the prevalence of STEC in one swine breeding farm in mainland China. STEC O157 was detected in 8 (1.1%) of 720 fecal samples, while non-O157 STEC was isolated from 33 (4.6%) samples (62). STEC strains were isolated from 10 (6%) of 169 swine fecal samples obtained from a number of swine farms in one province in Vietnam (63).

In addition to domestic swine, feral swine and wild boars have been suggested to play a role in STEC transmission. The most prominent evidence was obtained from a study that took place in the U.S. in 2006. Feral swine were suggested to be involved in the contamination of spinach, which was associated with a nationwide outbreak caused by STEC O157:H7. The outbreak strain was isolated from feral swine feces, which were collected directly on the spinach fields (29). Moreover, reports in other countries also indicated the presence of STEC in wild boars. STEC O157:H7 was found in 1 (1.4%) of 68 wild boars in Sweden (64). One Spanish study investigated the presence of STEC O157:H7 and non-O157 STEC in wild boars. STEC O157:H7 was detected in 7 (3.3%) and non-O157 STEC was detected in 11 (5.2%) fecal samples from 212 wild boars (65).

Human-animal contact is an important risk factor for STEC transmission, thus the presence of STEC in swine in zoos and petting zoos has also been investigated. No STEC O157 was detected in 45 pig fecal samples collected at U.S. institutions accredited by the Association of Zoos and Aquariums (14). In contrast, STEC O157:H7 was recovered in 13 (1.2%) of 1102 fecal samples from pigs in 19 county fairs in two states in the U.S (13). In the United Kingdom, an epidemiologic study was conducted to detect the presence of STEC O157 in "open farms" in which there is close contact with animals to attract the general public (66). STEC O157 was isolated in thirty (17.9%) of 168 swine fecal samples. However, this number may not reflect the true prevalence at one time point as it represents a collated number from samples collected from 22 different farms within a ten-year period. Moreover, it was not clear whether the pigs were housed together or in close contact with other animal species. Though the prevalence of STEC O157:H7 in these reports was low, pigs at facilities where direct contact with human beings may occur are another potential risk factor of STEC transmission.

In general, these data indicated that STEC strains in swine are geographically widely distributed given the fact that reports were from many regions in different continents. Although the prevalence numbers are wide ranging, these data provide epidemiologic evidence that STEC strains are prevalent in swine. Multiple factors may account for the discrepancies in the data among these studies, such as differences in farm management systems, sample sources, sampling methods, isolation protocols, and diagnostic tests used.

A number of reports have described detection of STEC O157 in swine fecal samples, and various non-O157 STEC serotypes were also detected in swine (41, 55, 56). STEC belonging to various serogroups of which some that have been associated with human illness were identified in swine (41, 56), and, surprisingly, none of the serogroups were those commonly associated

with swine edema disease. In addition, most of the above epidemiologic studies were cross-sectional studies, and they are unable to determine if the prevalence of STEC in swine changes with time (67). More research needs to be done to understand STEC shed by clinically healthy swine.

STEC IN SWINE: EDEMA DISEASE

Besides isolation from clinically healthy swine, STEC can cause edema disease, which affects post-weaning pigs and young finishing pigs. Some STEC strains, in particular edema disease-associated STEC, also play a role in fatal shock, that occurs in pre- and post-weaning pigs (68). STEC strains belonging to certain serogroups, including O138, O139, O141, and O147 are more frequently associated with edema disease (69). These STEC strains colonize in the small intestine and typically produce the variant Shiga toxin 2e (Stx2e). The toxin enters the bloodstream and binds to the specific receptor, globotetraosylceramide (Gb4), which is located on epithelial and endothelial cells (70). The toxin then impairs blood vessels, leading to edema, ataxia, and death (68). In general, the clinical presentations of swine edema disease are somewhat different from those associated with human STEC diseases.

Besides Stx2e, STEC strains associated with edema disease have been found to possess virulence factors different from those found in STEC strains isolated from human clinical cases. For example, F18 fimbriae are absent in most human-derived STEC (71), but are essential for adherence to swine epithelial cells (72), and therefore are likely to play a role in specificity and adaptation to the host. There are risk factors, including dietary changes and the introduction of pigs to new herds, suggested to be important for edema disease onset (68).

HUMAN STRAINS OF STX2E-PRODUCING *E. COLI*

Interestingly, in addition to their isolation from pigs, Stx2e-producing *E. coli* strains were recovered from samples of wastewater treatment plants in France (73), and from meat (pork, beef, wildlife) and milk in Germany (74). Notably, though rarely documented, Stx2e-producing *E. coli* strains have been isolated from human patients with HUS (75) and uncomplicated diarrhea (71, 76-79). Moreover, Stx2e-producing *E. coli* strains have been recovered from stool samples of asymptomatic human beings (71, 78). The etiologic role of Stx2e-producing *E. coli* strains in these human cases has not been determined. No particular source of infection has been identified in these human cases associated with Stx2e-producing *E. coli*.

A study was conducted to analyze Stx2e-producing *E. coli* strains from asymptomatic people, people with uncomplicated diarrhea, and diseased pigs to compare their virulence gene profiles and adherence to human and swine intestinal epithelial cells (71). Virulence genes commonly found in STEC strains associated with HC and HUS, such as the gene, *eae,* that encodes for intimin, an important attachment protein, were not detected in both the human- and swine-derived Stx2e-producing *E. coli* strains. This fact may suggest that unknown virulence factors are involved in the pathogenicity of human Stx2e-producing *E. coli* strains. Additionally, the human-derived Stx2e-producing *E. coli* strains adhered to human epithelial cells but not swine epithelial cells. In contrast, swine-derived Stx2e-producing *E. coli* strains lysed human epithelial cells and adhered to swine epithelial cells. This study only analyzed Stx2e-producing *E. coli* strains from diseased pigs, and thus, Stx2e-producing *E. coli* strains from healthy pigs need further examination.

Beutin and colleagues, however, have characterized Stx2e-producing *E. coli* from people with uncomplicated diarrhea, people with no clinical symptoms, diseased pigs, and healthy pigs at slaughter facilities to determine their serotypes, distribution of virulence genes, and Stx2e production (80). They found that Stx2e-producing *E. coli* strains from different sources were heterogeneous with regard to serotypes and virulence genes. In agreement with the results of Sonntag *et al*. (71), virulence genes commonly detected in STEC strains associated with HC and HUS, such as *eae* and *ehx*A, the latter of which encodes an enterohemolysin, were not detected by PCR in both the human- and swine-derived Stx2e-producing strains analyzed in this study. Additionally, the authors used an enzyme immunoassay to find that human-derived strains produced significantly higher amounts of Stx2e than strains from diseased pigs. Interestingly, the toxin-production level did not significantly differ between strains from human subjects with uncomplicated diarrhea and those with no clinical symptoms. However, different environments (in hosts or under experimental conditions) may contribute to the differences of toxin production. Another likely explanation is that there are other unidentified virulence factors in the pathogenic Stx2e-producing *E. coli* strains.

In all of these reports, the primary conclusion was that there was a lack of evidence to suggest that Stx2e-producing *E. coli* strains are a critical public health concern. Nevertheless, these results also suggest that unknown virulence factors may contribute to the pathogenesis of Stx2e-producing *E. coli* strains in human hosts. Additionally, Stx2e-producing *E. coli* strains have been mostly isolated from human patients with uncomplicated diarrhea. Patients with uncomplicated diarrhea may not seek medical attention, which may contribute to the low reporting frequencies of Stx2e-producing *E. coli* infections in human patients. Some commercial serological tests do not detect the toxin Stx2e (81). The limitation of diagnostic tests may

contribute to missed detection of Stx2e-producing *E. coli* infections. On the other hand, the picture remains unclear regarding the source of Stx2e-producing *E. coli* in human infections. More research is warranted to address whether there is an association between human Stx2e-producing *E. coli* strains and strains from pork, pigs, or the associated swine environment.

MOLECULAR EPIDEMIOLOGY OF SWINE STEC

The presence of virulence genes other than Shiga toxin genes (*stx*) also plays a role in the capability of STEC strains to cause disease (82). Therefore, examining the presence or absence of the selected virulence genes (virulence gene profile) is essential in assessing the public health risk of STEC strains (83, 84). A considerable challenge for understanding virulence gene profiles of swine STEC is that the profile of virulence genes targeted varies in each report. The most common virulence genes evaluated are *stx*1, *stx*2, *eae*, and *ehx*A. Different *stx*1, *stx*2, *eae*, *ehx*A gene combinations have been detected in swine STEC strains (45, 85). In the NAHMS 2000 study in the U.S., although a majority of swine STEC strains carried the *stx2*e gene, 6% of the strains carried other *stx*2 variants. The *eae* gene was not detected in any of the swine isolates in this study (41), and this was in agreement with other studies (58, 61). In contrast, many studies have identified the *eae* gene in STEC strains from clinically healthy swine (13, 33, 36, 45, 50-53, 55-57, 59, 85). Meanwhile, detection of the *ehx*A gene in swine STEC isolates was reported in numerous studies (36, 41, 50, 51, 55, 56, 59, 65, 86). A limited number of studies described testing for different subsets of STEC virulence genes in swine STEC strains (41, 50, 86, 87). For instance, some *eae*-negative swine STEC strains carried the *saa* gene (STEC autoagglutinating adhesion), and other adhesins (86). The above studies suggest that swine STEC strains carry various combinations of virulence genes. A subset of swine STEC strains does carry important virulence genes in human pathogenesis, such as *eae*. More extensive examination is needed because the presence or absence of many other STEC virulence genes has not been examined in swine STEC strains.

Molecular genotyping methods, such as pulsed-field gel electrophoresis (PFGE), have been frequently utilized to evaluate the pathogenic potential of STEC strains and provide suggestions for future strategies in control and prevention of STEC transmission (88, 89). For example, these methods are used to identify the source of STEC during food-borne outbreaks and to determine the genetic relatedness of STEC strains (90-94). A limited number of studies have incorporated a small number of swine STEC strains to examine their genetic relatedness with strains from human subjects with diarrhea, HC, HUS, or without clinical symptoms (46, 53, 95, 96). Most of these studies focused on STEC O157:H7 strains, and only one study investigated STEC O101 strains (95). The results from these studies were inconsistent. One Chilean study indicated that the swine STEC O157 strains were categorized into the same cluster with strains from human HUS cases in the same geographic area by analyzing the PFGE patterns. However, no further epidemiologic relationship could be drawn between the swine isolates and human cases (46). One study conducted in Norway reported similarities of PFGE patterns among swine STEC O157:H7 strains and those isolated from human cases. However, there was no other epidemiologic information provided about the human STEC cases (53). In contrast, in an Irish study the investigators stated that swine STEC O157:H7 strains were not genetically related to human strains at the PFGE pattern similarity criterion of 80% (96).

One study implemented repetitive element sequence-based PCR (rep-PCR) to examine the genetic relatedness of STEC O101 strains from swine and a human case of diarrhea. Interestingly, the result of the analysis indicated that swine STEC O101 strains showed a high degree of relatedness with the human STEC O101 strain. Moreover, the *stx*2e gene sequences from STEC O101 strains of two different origins shared a high degree of homology, and none of the STEC O101 strains carried the *eae* or *ehx*A genes (95). A direct relationship cannot be

ascertained between the swine and human STEC since the strains were from two different countries and isolated in different time periods. However, the molecular evidence highlights that swine may be a potential STEC reservoir and a source of human infections.

A number of studies have examined the genetic relatedness of swine STEC strains to strains isolated from other animal reservoirs. The PFGE patterns indicated that swine STEC strains were not closely related to STEC strains of bovine origin (46, 96), ovine origin (96) and from turkeys (51). A Korean study demonstrated that swine STEC O157:H7 strains had distinct randomly amplified polymorphic DNA (RAPD) patterns compared with strains from cattle at a similarity criterion of 63% (59). Another study showed that swine STEC O157:H7 strains demonstrated some similarity in PFGE patterns to strains from cattle (53). A Polish study reported that one swine STEC O157 strain clustered with STEC O157 strains from cattle at a similarity criterion of 80% based on PFGE patterns. The swine STEC O157 strain was from a weaned pig with diarrhea, and no other epidemiologic relationship was described between the swine and cattle STEC strains (97).

In the epidemiologic investigation following a spinach-associated outbreak of STEC O157:H7 in 2006 in the U.S., PFGE and multilocus variable number tandem repeat analysis (MLVA) were used to analyze STEC isolates from different sources (29). Indistinguishable PFGE patterns from STEC isolates from cattle, feral swine, surface water, and soil were identified. MLVA also categorized the STEC isolates with indistinguishable PFGE patterns together with the outbreak STEC strain as one cluster. This evidence led the authors to suggest that feral swine may serve as a risk factor for STEC contamination of the spinach fields (29). Inspired by this outbreak, a Spanish study used PFGE to examine the genetic relatedness of STEC isolates from wild boar and a human patient with diarrhea (65). Surprisingly, a STEC

isolate from a wild boar shared an indistinguishable PFGE pattern with a STEC isolate from a human patient with diarrhea in the same geographic area. Though there were no epidemiologic data available to establish a relationship between the human case and wild boar, the molecular genotyping results indicated that wild boars are a potential source of STEC contamination (65).

Results presented by these molecular epidemiologic studies are varied, which is potentially due to multiple factors. For example, there is potential selection bias of the isolates included in the genotypic analysis. Limited numbers of swine STEC strains and strains from other origins were included in the above molecular genotypic studies. The analytical results may not reflect the complete picture of genetic relatedness between STEC strains from swine and other origins. Moreover, most of the studies focused on STEC O157:H7. There is a paucity of knowledge on the genetic relatedness of non-O157 STEC from different origins. Conclusively, the genetic relationship of swine STEC strains with human clinical strains is still poorly understood. Filling these current knowledge gaps is relevant to not only better understand swine STEC but also to assess the potential public health risk of swine STEC.

THE EFFECT OF SWINE STEC STRAINS ON HUMAN INTESTINAL CELLS

Kennedy *et al.* (98) infected human Caco-2 cells, which is a human colonic epithelial cell line, with STEC O157:H7 strains isolated from human patients or from healthy pigs at slaughter facilities. The human and swine STEC strains possessed the same virulence gene profiles, including *eae* and other virulence genes, published in an earlier study (96). Notably, swine STEC O157:H7 strains induced greater loss of monolayer cell integrity than human-source STEC. Moreover, microarray experiments that examined RNA levels transcribed from different STEC genes indicated that swine and human STEC strains had different gene expression profiles. When exposed to swine STEC strains, expression levels of some genes involved in cytoskeleton rearrangement were up-regulated in the human Caco-2 cells (98). However, more research is needed to further examine the effect of infection with swine STEC strains on human epithelial cells.

STEC INFECTION IN SWINE: ANIMAL MODEL STUDIES

Swine have been used as an animal model in studies examining STEC colonization (99), virulence of different STEC strains (100, 101), and treatment of STEC infections (102). Interestingly, some studies evaluated the pathogenicity and colonization of intimin-negative STEC strains in swine models. One study found that intimin-negative non-O157 STEC strains colonize and cause similar intestinal lesions and systemic diseases as intimin-positive STEC O157:H7 strains in cesarean-derived colostrum-deprived (CDCD) neonatal pigs (103). Moreover, a later study reported that there were no significant differences in bacterial counts and the numbers of tissues infected between intimin-positive and intimin-negative STEC O157:H7 strains in 12-week-old adult pigs (104). Both intimin-positive and intimin-negative experimental strains were recovered in the pigs 38 days after inoculation. Notably, the result of this study in pigs is different from a previous study indicating that intimin aids the persistence of STEC O157:H7 strains in adult cattle and sheep (105). This may suggest that other adhesins contribute to the colonization of STEC in swine. The above evidence demonstrates that swine are biologically capable of carrying STEC, including intimin-positive STEC strains.

To further understand if swine have the potential to become reservoirs of STEC, researchers have pursued experimental challenge models. Booher and colleagues inoculated three-month-old pigs with mixtures of STEC O157:H7 and other pathogenic *E. coli* strains to observe the level and duration of fecal shedding (106). They reported that STEC O157:H7 strains were isolated from swine fecal samples up to two months after inoculation, and the experimental strains were recovered in swine intestinal tissues. Shedding of experimental STEC strains was dose-dependent, and strains inoculated at lower levels (10^7 CFU/strain/animal) were

not recovered in the feces two months after inoculation. The dose-dependent shedding is similar to what was found in cattle under experimental conditions (107). Interestingly, one of the experimental strains inoculated at the lower dose was recovered in swine cecum tissues at necropsy two months after inoculation (106). This study suggested that swine could shed STEC O157:H7 for at least two months.

Cornick and Helgerson conducted an experimental study to observe the transmission of STEC O157:H7 among pigs (108). The three-month-old pigs were inoculated with a STEC O157:H7 strain that originated from a human outbreak, via addition of the organisms added to their feed. Moreover, they were interested in examining the effect of two different housing conditions on STEC O157:H7 shedding level and duration. In this housing experiment, a STEC strain of bovine origin was inoculated in conjunction with the STEC strain of human origin. One group of pigs was housed individually on decks with feed provided from a trough, while the other groups of pigs were housed two per pen with feed given on the cement floors. Transmission of STEC O157:H7 was observed from infected donor pigs to naïve pigs, which were housed in the same pen. Fecal shedding of STEC O157:H7 was observed in exposed naïve pigs for at least two weeks after exposure. In addition, the level and duration of STEC O157:H7 shedding was not significantly affected by different housing conditions.

The above experimental study (108) supported a previous study (106), suggesting that shedding of STEC is dose-dependent and that swine are biologically competent hosts for STEC O157:H7. Additionally, results of this study (108) indicated that STEC O157:H7 infection may be maintained in the swine population and that transmission readily occurs between animals. In another subsequent study by the same research group, STEC O157:H7 was recovered not only

from pigs housed close to the infected donor in the adjacent pen (nose-to-nose contact), but also from naïve pigs housed in pens apart from the infected donor (109). The isolation of the experimental bacterial strain in the air samples provided further evidence to support that contaminated aerosols are a means of indirect STEC O157:H7 transmission among pigs.

Antibiotic-free feed was provided in the above animal model studies. However, in most conventional swine production systems, at least in the U.S., feed with antimicrobials is commonly used. Thus, the effect of antimicrobials in feed on STEC O157:H7 shedding was also examined (110). A number of frequently used antimicrobials for growth promotion in U.S. swine production were selected for this study, namely bacitracin methylene disalicylate, chlortetracycline, and tylosin. Three groups of young adult pigs were given feed with each of the three selected antimicrobials, and another group of control pigs was fed with antimicrobial-free feed. A STEC O157:H7 strain from a human outbreak in Washington State in the U.S. (111) was orally inoculated at a dose of 10^7 CFU/strain/animal. One month after inoculation, the numbers of pigs that shed STEC O157:H7 and levels of STEC O157:H7 shedding were significantly lower in two groups given feed with tylosin and chlortetracycline compared to the control group. However, no significant differences were found in levels and numbers of pigs that shed STEC O157:H7 between the bacitracin and control groups. Interestingly, a more recent study by Wells *et al*. (42) reported the numbers of pigs shedding STEC O26, O103, and O145 did not significantly differ between antimicrobial and control groups. More research is needed to evaluate the effect of sub-therapeutic doses of antimicrobials in feed on STEC shedding.

FUTURE DIRECTIONS

Swine are not viewed as an important STEC reservoir given the rare known incidence of cases of severe human illness associated with STEC of swine origin. Nevertheless, the association between swine STEC and human illnesses needs to be further investigated. First of all, pork products and feral swine have been associated with a number of STEC outbreaks. STEC causes edema disease in swine. However, STEC can be also isolated from clinically healthy pigs at rates occasionally higher than those for cattle. In addition, animal model studies suggested that swine are biologically competent for colonization and for shedding of STEC for at least two months after inoculation.

The increasing surveillance of non-O157 STEC infections has raised the awareness of non-O157 STEC (20, 112-115). In the U. S., six non-O157 STEC serogroups, namely O26, O103, O111, O121, O45, O145, were recently classified as adulterants, similar to O157, in raw, non-intact beef product (116). Non-O157 serogroups, namely O26, O103, O145, O111, and O91, were also identified as major public health concerns in Europe (117). Some of the non-O157 STEC strains were highly virulent and carried virulence gene profiles which were rarely reported in previous studies. For example, the STEC O104:H4 strain involved in a large-scale outbreak in 2011 in Germany was an enteroaggregative *E. coli* that carried genes typical of this pathogenic *E. coli* group, but that also carried the *stx*2 gene (118). Most STEC strains detected in clinically healthy swine were non-O157 STEC (41). However, there is still very limited information about swine STEC.

Based on the increasing role of non-O157 STEC in human illnesses, the high prevalence and limited epidemiologic investigation of STEC in swine, we recommend further research to

elucidate the unclear association between swine STEC and human illness. This will serve public health through determining whether swine are an important reservoir for STEC infection in people. If the evidence indicates swine are an important reservoir, efforts can be directed at control of this pathogen in this species, and, if swine ultimately are determined to not be an important reservoir, public health resources can be directed away from swine and towards further understanding of the epidemiology of STEC, particularly non-O157 serotypes.

APPENDIX

Table 1.1. Outbreaks associated with pork products

Outbreak location and year	STEC serotype	*stx* gene subtype	*eae* gene	food product	reference
Washington and California, U.S., 1994	O157:H7	not specified	not specified	salami (mixed pork and beef)	(22)
Australia, 1995	O111:H-	*stx*1, *stx*2	positive	mettwurst (mixture of raw pork, beef and lamb)	(23, 24)
Ontario, Canada, 1998	O157:H7	not specified	not specified	salami (mixed pork and beef)	(25)(Williams *et al.*, 2000)
British Columbia, Canada, 1999	O157:H7	not specified	not specified	salami (mixed pork and beef)	(26)

Table 1.1. (cont'd)

Outbreak location and year	STEC serotype	*stx* gene subtype	*eae* gene	food product	reference
Italy, 2004	O157:non-motile	*stx*1, *stx*2	positive	salami (pork only)	(27)
Ontario, Canada, 2011	O157:H7	not specified	not specified	pork from a whole roasted pig	(28)

REFERENCES

REFERENCES

1. Karmali, M.A., et al., *The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing Escherichia coli.* J Infect Dis, 1985. **151**(5): p. 775-82.

2. Nataro, J.P. and J.B. Kaper, *Diarrheagenic Escherichia coli.* Clin Microbiol Rev, 1998. **11**(1): p. 142-201.

3. Karmali, M.A., *Infection by verocytotoxin-producing Escherichia coli.* Clin Microbiol Rev, 1989. **2**(1): p. 15-38.

4. Tarr, P.I., C.A. Gordon, and W.L. Chandler, *Shiga-toxin-producing Escherichia coli and haemolytic uraemic syndrome.* The Lancet, 2005. **365**(9464): p. 1073-1086.

5. Williams, D.M., et al., *Acute kidney failure: a pediatric experience over 20 years.* Arch Pediatr Adolesc Med, 2002. **156**(9): p. 893-900.

6. Karmali, M.A., V. Gannon, and J.M. Sargeant, *Verocytotoxin-producing Escherichia coli (VTEC).* Vet Microbiol, 2010. **140**(3-4): p. 360-70.

7. Scallan, E., et al., *Foodborne Illness Acquired in the United States—Major Pathogens.* Emerging Infectious Diseases, 2011. **17**(1): p. 7-15.

8. Hoffmann, S., M.B. Batz, and J.G. Morris, *Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens.* Journal of Food Protection, 2012. **75**(7): p. 1292-1302.

9. Rangel, J.M., et al., *Epidemiology of Escherichia coli O157:H7 outbreaks, United States, 1982-2002.* Emerg Infect Dis, 2005. **11**(4): p. 603-9.

10. Doyle, M.P., Archer J, Kaspar CW, Weiss R *Human illness casued by E. coli O157:H7 from food and non-food sources* 2006 02/23/2012 Feburary, 2013]; Available from: http://fri.wisc.edu/docs/pdf/FRIBrief_EcoliO157H7humanillness.pdf.

11. Kaspar, C., Doyle ME, Archer J. *White paper on non-O157:H7 Shiga toxin-producing E. coli from meat and non-meat sources* 2010 02/20/2012 Feburary 2013]; Available from: http://fri.wisc.edu/docs/pdf/FRI_Brief_NonO157STEC_4_10.pdf.

12. Durso, L.M., et al., *Shiga-toxigenic Escherichia coli O157:H7 infections among livestock exhibitors and visitors at a Texas County Fair.* Vector Borne and Zoonotic Diseases, 2005. **5**(2): p. 193-201.

13. Keen, J.E., et al., *Shiga-toxigenic Escherichia coli O157 in agricultural fair livestock, United States.* Emerg Infect Dis, 2006. **12**(5): p. 780-6.

14. Keen, J.E., L.M. Durso, and T.P. Meehan, *Isolation of Salmonella enterica and Shiga-toxigenic Escherichia coli O157 from feces of animals in public contact areas of United States zoological parks.* Appl Environ Microbiol, 2007. **73**(1): p. 362-5.

15. Spika, J.S., et al., *Hemolytic uremic syndrome and diarrhea associated with Escherichia coli O157:H7 in a day care center.* J Pediatr, 1986. **109**(2): p. 287-91.

16. Carter, A.O., et al., *A severe outbreak of Escherichia coli O157:H7--associated hemorrhagic colitis in a nursing home.* N Engl J Med, 1987. **317**(24): p. 1496-500.

17. Rowe, P.C., et al., *Diarrhoea in close contacts as a risk factor for childhood haemolytic uraemic syndrome. The CPKDRC co-investigators.* Epidemiol Infect, 1993. **110**(1): p. 9-16.

18. Varma, J.K., et al., *An outbreak of Escherichia coli O157 infection following exposure to a contaminated building.* The Journal of Aermican Medical Association, 2003. **290**(20): p. 2709-12.

19. Griffin, P.M. and R.V. Tauxe, *The epidemiology of infections caused by Escherichia coli O157:H7, other enterohemorrhagic E. coli, and the associated hemolytic uremic syndrome.* Epidemiol Rev, 1991. **13**: p. 60-98.

20. Gyles, C.L., *Shiga toxin-producing Escherichia coli: an overview.* J Anim Sci, 2007. **85**(13 Suppl): p. E45-62.

21. Dean-Nystrom, E.A., et al., *Pathogenicity of Escherichia coli O157:H7 in the intestines of neonatal calves.* Infection and Immunity, 1997. **65**(5): p. 1842-8.

22. CDC, *Escherichia coli O157:H7 outbreak linked to commercially distributed dry-cured salami- Washington and California, 1994.* Morbidity and Mortality Weekly Report, 1995. **44**: p. 157-160.

23. CDC, *Community outbreak of hemolytic uremic syndrome attributable to Escherichia coli O111:NM-South Australia, 1995.* Morbidity and Mortality Weekly Report, 1995. **44**: p. 550-558.

24. Paton, A.W., et al., *Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing Escherichia coli.* J Clin Microbiol, 1996. **34**(7): p. 1622-7.

25. Williams, R.C., et al., *Illness outbreak associated with Escherichia coli O157:H7 in Genoa salami. E. coli O157:H7 Working Group.* Canadian Medical Association Journal, 2000. **162**(10): p. 1409-13.

26. MacDonald, D.M., et al., *Escherichia coli O157:H7 outbreak linked to salami, British Columbia, Canada, 1999.* Epidemiol Infect, 2004. **132**(2): p. 283-9.

27. Conedera, G., et al., *A family outbreak of Escherichia coli O157 haemorrhagic colitis caused by pork meat salami.* Epidemiol Infect, 2007. **135**(2): p. 311-4.

28. Trotz-Williams, L.A., et al., *Pork Implicated in a Shiga Toxin-producing Escherichia coli O157:H7 Outbreak in Ontario, Canada.* Canadian Journal of Public Health. Revue Canadienne de Sante Publique, 2012. **103**(5): p. e322-6.

29. Jay, M.T., et al., *Escherichia coli O157:H7 in feral swine near spinach fields and cattle, central California coast.* Emerg Infect Dis, 2007. **13**(12): p. 1908-11.

30. Karmali, M.A., et al., *The serotype and biotype distribution of clinical isolates of Campylobacter jejuni and Campylobacter coli over a three-year period.* Journal of Infectious Diseases, 1983. **147**(2): p. 243-6.

31. Samadpour, M., et al., *Occurrence of Shiga-like toxin-producing Escherichia coli in retail fresh seafood, beef, lamb, pork, and poultry from grocery stores in Seattle, Washington.* Appl Environ Microbiol, 1994. **60**(3): p. 1038-40.

32. Brooks, H.J., et al., *Occurrence and virulence factors of non-O157 Shiga toxin-producing Escherichia coli in retail meat in Dunedin, New Zealand.* Lett Appl Microbiol, 2001. **32**(2): p. 118-22.

33. Jo, M.Y., et al., *Prevalence and characteristics of Escherichia coli O157 from major food animals in Korea.* Int J Food Microbiol, 2004. **95**(1): p. 41-9.

34. Oteiza, J.M., et al., *Isolation and characterization of Shiga toxin-producing Escherichia coli from precooked sausages (morcillas).* Food Microbiology, 2006. **23**(3): p. 283-8.

35. Xia, X., et al., *Presence and characterization of Shiga toxin-producing Escherichia coli and other potentially diarrheagenic E. coli strains in retail meats.* Appl Environ Microbiol, 2010. **76**(6): p. 1709-17.

36. Ateba, C.N. and M. Mbewe, *Detection of Escherichia coli O157:H7 virulence genes in isolates from beef, pork, water, human and animal species in the northwest province, South Africa: public health implications.* Res Microbiol, 2011. **162**(3): p. 240-248.

37. Li, M.C., F. Wang, and F. Li, *Identification and molecular characterization of antimicrobial-resistant Shiga toxin-producing Escherichia coli isolated from retail meat products.* Foodborne Pathogens and Disease, 2011. **8**(4): p. 489-93.

38. Feder, I., et al., *Testing of swine feces obtained through the National Animal Health Monitoring System's Swine 2000 study for the presence of Escherichia coli O157:H7.* J Food Prot, 2007. **70**(6): p. 1489-92.

39. Bush, E., *U.S. swine herd appears free of Escherichia coli O157:H7.* Food Safety Digest, 1997. **4**.

40. Fratamico, P.M., et al., *Prevalence and characterization of Shiga toxin-producing Escherichia coli in swine feces recovered in the National Animal Health Monitoring System's Swine 2000 study.* Appl Environ Microbiol, 2004. **70**(12): p. 7173-8.

41. Fratamico, P.M., et al., *Characterization of Shiga toxin-producing Escherichia coli strains isolated from swine feces.* Foodborne Pathogen and Disease, 2008. **5**(6): p. 827-38.

42. Wells, J.E., et al., *Effects of antimicrobials fed as dietary growth promoters on fecal shedding of Campylobacter, Salmonella, and Shiga-toxin producing Escherichia coli in swine.* Journal of Applied Microbiology, 2012. **114**(2): p. 318-328.

43. Oporto, B., et al., *Escherichia coli O157:H7 and non-O157 Shiga toxin-producing E. coli in healthy cattle, sheep and swine herds in Northern Spain.* Zoonoses Public Health, 2008. **55**(2): p. 73-81.

44. Pinaka, O., et al., *Shiga toxin-producing Escherichia coli in Central Greece: prevalence and virulence genes of O157:H7 and non-O157 in animal feces, vegetables, and humans.* European Journal of Clinical Microbiology and Infectious Diseases, 2013.

45. Borie, C., et al., *Prevalence and characterization of enterohaemorrhagic Escherichia coli isoated from healthy cattle and pigs slaughtered in Santiago, Chile.* Archivos De Medicina Veterinaria, 1997. **29**(2): p. 205-212.

46. Rios, M., et al., *Clonal diversity of Chilean isolates of enterohemorrhagic Escherichia coli from patients with hemolytic-uremic syndrome, asymptomatic subjects, animal reservoirs, and food products.* J Clin Microbiol, 1999. **37**(3): p. 778-81.

47. Martins, R.P., et al., *Prevalence of enterotoxigenic and Shiga toxin-producing Escherichia coli in pigs slaughtered in Mato Grosso, Brazil.* J Infect Dev Ctries, 2011. **5**(2): p. 123-7.

48. Borges, C.A., et al., *Shiga Toxigenic and Atypical Enteropathogenic Escherichia coli in the Feces and Carcasses of Slaughtered Pigs.* Foodborne Pathogens and Disease, 2012. **9**(12): p. 1119-1125.

49. Milnes, A.S., et al., *Factors related to the carriage of Verocytotoxigenic E. coli, Salmonella, thermophilic Campylobacter and Yersinia enterocolitica in cattle, sheep and pigs at slaughter.* Epidemiol Infect, 2009. **137**(8): p. 1135-48.

50. Lenahan, M., et al., *The potential use of chilling to control the growth of Enterobacteriaceae on porcine carcasses and the incidence of E. coli O157:H7 in pigs.* J Appl Microbiol, 2009. **106**(5): p. 1512-20.

51. Heuvelink, A.E., et al., *Isolation and characterization of verocytotoxin-producing Escherichia coli O157 from slaughter pigs and poultry.* Int J Food Microbiol, 1999. **52**(1-2): p. 67-75.

52. Bonardi, S., et al., *Detection of Salmonella spp., Yersinia enterocolitica and verocytotoxin-producing Escherichia coli O157 in pigs at slaughter in Italy.* Int J Food Microbiol, 2003. **85**(1-2): p. 101-10.

53. Johnsen, G., et al., *Escherichia coli O157:H7 in faeces from cattle, sheep and pigs in the southwest part of Norway during 1998 and 1999.* Int J Food Microbiol, 2001. **65**(3): p. 193-200.

54. Eriksson, E., et al., *Verocytotoxin-producing Escherichia coli O157:H7 in the Swedish pig population.* Vet Rec, 2003. **152**(23): p. 712-7.

55. Botteldoorn, N., et al., *Detection and characterization of verotoxigenic Escherichia coli by a VTEC/EHEC multiplex PCR in porcine faeces and pig carcass swabs.* Res Microbiol, 2003. **154**(2): p. 97-104.

56. Kaufmann, M., et al., *Escherichia coli O157 and non-O157 Shiga toxin-producing Escherichia coli in fecal samples of finished pigs at slaughter in Switzerland.* J Food Prot, 2006. **69**(2): p. 260-6.

57. Nakazawa, M. and M. Akiba, *Swine as a potential reservoir of Shiga toxin-producing Escherichia coli O157:H7 in Japan.* Emerg Infect Dis, 1999. **5**(6): p. 833-4.

58. Kijima-Tanaka, M., et al., *A national surveillance of Shiga toxin-producing Escherichia coli in food-producing animals in Japan.* Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health, 2005. **52**(5): p. 230-7.

59. Kim, J., et al., *Isolation and identification of Escherichia coli O157:H7 using different detection methods and molecular determination by multiplex PCR and RAPD.* Journal of Veterinary Science, 2005. **6**(1): p. 7-19.

60. Keum, H.O., Kim HK, Rho SM, Moon HJ, Park SJ and Park BK, *Comparative study of Clostridium perfringens, Salmonella spp. and E.coli focused on characteristics of E. coli O157 isolated from pigs of HACCP- and non-HACCP-accredited swine farms in Korea.* Korean Journal of Veterinary Research, 2010. **50**(2): p. 113-116.

61. Leung, P.H., et al., *The prevalence and characterization of verotoxin-producing Escherichia coli isolated from cattle and pigs in an abattoir in Hong Kong.* Epidemiol Infect, 2001. **126**(2): p. 173-9.

62. Yan, Y., et al., *Prevalence of Stx phages in environments of a pig farm and lysogenic infection of the field E. coli O157 isolates with a recombinant converting phage.* Curr Microbiol, 2011. **62**(2): p. 458-64.

63. Kobayashi, H., et al., *Prevalence of pathogenic Escherichia coli in a swine breeding environment in Can Tho Province, Vietnam.* JARQ, Japan Agricultural Research Quarterly, 2003. **37**(1): p. 59-63.

64. Wahlstrom, H., et al., *Survey of Campylobacter species, VTEC O157 and Salmonella species in Swedish wildlife.* Vet Rec, 2003. **153**(3): p. 74-80.

65. Sánchez, S., et al., *Detection and characterisation of O157:H7 and non-O157 Shiga toxin-producing Escherichia coli in wild boars.* Veterinary Microbiology, 2010. **143**(2-4): p. 420-423.

66. Pritchard, G.C., et al., *Verocytotoxigenic Escherichia coli O157 in animals on public amenity premises in England and Wales, 1997 to 2007.* Vet Rec, 2009. **164**(18): p. 545-9.

67. Dohoo, I., Martin W, Stryhn H, *Cross-sectional studies*, in *Veterinary Epidemiologic Research*. 2010, VER Inc.: Charlottetown, Prince Edward Island, Canada. p. 158-162.

68. Fairbrother, J.M., Gyles CL, *Colibacillosis*, in *Diseases of Swine*, L.K. Jeffrey Zimmerman, Alejandro Ramirez, Kent Schwartz,Gregory Stevenson Editor. 2012, John Wiley and sons, Inc.: West Sussex, UK. p. 723-747.

69. Fairbrother, J.M., E. Nadeau, and C.L. Gyles, *Escherichia coli in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies.* Anim Health Res Rev, 2005. **6**(1): p. 17-39.

70. DeGrandis, S., et al., *Globotetraosylceramide is recognized by the pig edema disease toxin.* Journal of Biological Chemistry, 1989. **264**(21): p. 12520-5.

71. Sonntag, A.K., et al., *Shiga toxin 2e-producing Escherichia coli isolates from humans and pigs differ in their virulence profiles and interactions with intestinal epithelial cells.* Appl Environ Microbiol, 2005. **71**(12): p. 8855-63.

72. Bertschinger, H.U., Gyles CL *Oedema disease of pigs*, in *Escherichia coli in doemstic animals humans*, C.L. Gyles, Editor. 1994, CAB International: Wallingofrd, UK. p. 193-219.

73. Vernozy-Rozand, C., et al., *Serotyping, stx2 subtyping, and characterization of the locus of enterocyte effacement island of Shiga toxin-producing Escherichia coli and E. coli O157:H7 strains isolated from the environment in France.* Appl Environ Microbiol, 2004. **70**(4): p. 2556-9.

74. Beutin, L., et al., *Identification of human-pathogenic strains of Shiga toxin-producing Escherichia coli from food by a combination of serotyping and molecular typing of Shiga toxin genes.* Appl Environ Microbiol, 2007. **73**(15): p. 4769-75.

75. Thomas, A., et al., *Isolation of Vero cytotoxin-producing Escherichia coli serotypes O9ab:H- and O101:H-carrying VT2 variant gene sequences from a patient with haemolytic uraemic syndrome.* Eur J Clin Microbiol Infect Dis, 1994. **13**(12): p. 1074-6.

76. Pierard, D., et al., *Diarrhoea associated with Escherichia coli producing porcine oedema disease verotoxin.* Lancet, 1991. **338**(8769): p. 762.

77. Muniesa, M., et al., *Characterization of a Shiga toxin 2e-converting bacteriophage from an Escherichia coli strain of human origin.* Infect Immun, 2000. **68**(9): p. 4850-5.

78. Friedrich, A.W., et al., *Escherichia coli harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms.* J Infect Dis, 2002. **185**(1): p. 74-84.

79. Beutin, L., et al., *Characterization of Shiga toxin-producing Escherichia coli strains isolated from human patients in Germany over a 3-year period.* J Clin Microbiol, 2004. **42**(3): p. 1099-108.

80. Beutin, L., et al., *Evaluation of major types of Shiga toxin 2e-producing Escherichia coli bacteria present in food, pigs, and the environment as potential pathogens for humans.* Applied and Environmental Microbiology, 2008. **74**(15): p. 4806-4816.

81. Feng, P.C., et al., *Specificity of PCR and serological assays in the detection of Escherichia coli Shiga toxin subtypes.* Applied and Environmental Microbiology, 2011. **77**(18): p. 6699-702.

82. Bugarel, M., et al., *Virulence gene profiling of enterohemorrhagic (EHEC) and enteropathogenic (EPEC) Escherichia coli strains: a basis for molecular risk assessment of typical and atypical EPEC strains.* BMC Microbiol, 2011. **11**: p. 142.

83. Cobbold, R. and P. Desmarchelier, *A longitudinal study of Shiga-toxigenic Escherichia coli (STEC) prevalence in three Australian diary herds.* Vet Microbiol, 2000. **71**(1-2): p. 125-37.

84. Slanec, T., et al., *Molecular analysis of virulence profiles and Shiga toxin genes in food-borne Shiga toxin-producing Escherichia coli.* Appl Environ Microbiol, 2009. **75**(19): p. 6187-97.

85. Feder, I., et al., *Isolation of Escherichia coli O157:H7 from intact colon fecal samples of swine.* Emerg Infect Dis, 2003. **9**(3): p. 380-3.

86. Chapman, T.A., et al., *Comparison of virulence gene profiles of Escherichia coli strains isolated from healthy and diarrheic swine.* Appl Environ Microbiol, 2006. **72**(7): p. 4782-95.

87. Zweifel, C., et al., *Virulence profiles of Shiga toxin 2e-producing Escherichia coli isolated from healthy pig at slaughter.* Vet Microbiol, 2006. **117**(2-4): p. 328-32.

88. Cobbold, R. and P. Desmarchelier, *Characterisation and clonal relationships of Shiga-toxigenic Escherichia coli (STEC) isolated from Australian dairy cattle.* Vet Microbiol, 2001. **79**(4): p. 323-35.

89. Lanier, W.A., et al., *Pulsed-field gel electrophoresis subtypes of Shiga toxin-producing Escherichia coli O157 isolated from ground beef and humans, United States, 2001-2006.* Foodborne Pathogens and Disease, 2009. **6**(9): p. 1075-82.

90. Barrett, T.J., et al., *Laboratory investigation of a multistate food-borne outbreak of Escherichia coli O157:H7 by using pulsed-field gel electrophoresis and phage typing.* J Clin Microbiol, 1994. **32**(12): p. 3013-7.

91. Samadpour, M., *Molecular epidemiology of Escherichia coli O157:H7 by restriction fragment length polymorphism using Shiga-like toxin genes.* J Clin Microbiol, 1995. **33**(8): p. 2150-4.

92. Diekema, D.J., et al., *A cluster of serious Escherichia coli infections in a neonatal intensive-care unit.* Infect Control Hosp Epidemiol, 1997. **18**(11): p. 774-6.

93. Izumiya, H., et al., *Molecular typing of enterohemorrhagic Escherichia coli O157:H7 isolates in Japan by using pulsed-field gel electrophoresis.* J Clin Microbiol, 1997. **35**(7): p. 1675-80.

94. Singer, R.S., W.M. Sischo, and T.E. Carpenter, *Exploration of biases that affect the interpretation of restriction fragment patterns produced by pulsed-field gel electrophoresis.* J Clin Microbiol, 2004. **42**(12): p. 5502-11.

95. Franke, S., et al., *Clonal relatedness of Shiga-like toxin-producing Escherichia coli O101 strains of human and porcine origin.* J Clin Microbiol, 1995. **33**(12): p. 3174-8.

96. Lenahan, M., et al., *Molecular characterization of IrishE. coliO157:H7 isolates of human, bovine, ovine and porcine origin.* Journal of Applied Microbiology, 2009. **107**(4): p. 1340-1349.

97. Osek, J. and P. Gallien, *Molecular analysis of Escherichia coli O157 strains isolated from cattle and pigs by the use of PCR and pulsed-field gel electrophoresis methods.* Veterinarni Medicina, 2002. **47**(6): p. 149-158.

98. Kennedy, C.A., et al., *Shiga toxin-producing Escherichia coli isolated from human and pig origin induce different gene expression profiles in human Caco-2 epithelial cells.* Livestock Science, 2010. **133**(1/3): p. 189-191.

99. Jordan, D.M., et al., *Long polar fimbriae contribute to colonization by Escherichia coli O157:H7 in vivo.* Infect Immun, 2004. **72**(10): p. 6168-71.

100. Baker, D.R., et al., *Differences in Virulence among Escherichia coli O157:H7 Strains Isolated from Humans during Disease Outbreaks and from Healthy Cattle.* Applied and Environmental Microbiology, 2007. **73**(22): p. 7338-7346.

101. Shringi, S., et al., *Differential virulence of clinical and bovine-biased enterohemorrhagic Escherichia coli O157:H7 genotypes in piglet and Dutch belted rabbit models.* Infect Immun, 2012. **80**(1): p. 369-80.

102. Sheoran, A.S., et al., *Human antibody against Shiga toxin 2 administered to piglets after the onset of diarrhea due to Escherichia coli O157:H7 prevents fatal systemic complications.* Infect Immun, 2005. **73**(8): p. 4607-13.

103. Dean-Nystrom, E.A., et al., *Comparative pathogenicity of Escherichia coli O157 and intimin-negative non-O157 Shiga toxin-producing E coli strains in neonatal pigs.* Infect Immun, 2003. **71**(11): p. 6526-33.

104. Jordan, D.M., S.L. Booher, and H.W. Moon, *Escherichia coli O157:H7 does not require intimin to persist in pigs.* Infect Immun, 2005. **73**(3): p. 1865-7.

105. Cornick, N.A., S.L. Booher, and H.W. Moon, *Intimin facilitates colonization by Escherichia coli O157:H7 in adult ruminants.* Infect Immun, 2002. **70**(5): p. 2704-7.

106. Booher, S.L., N.A. Cornick, and H.W. Moon, *Persistence of Escherichia coli O157:H7 in experimentally infected swine.* Vet Microbiol, 2002. **89**(1): p. 69-81.

107. Cray, W.C., Jr. and H.W. Moon, *Experimental infection of calves and adult cattle with Escherichia coli O157:H7.* Applied and Environmental Microbiology, 1995. **61**(4): p. 1586-90.

108. Cornick, N.A. and A.F. Helgerson, *Transmission and infectious dose of Escherichia coli O157:H7 in swine.* Appl Environ Microbiol, 2004. **70**(9): p. 5331-5.

109. Cornick, N.A. and H. Vukhac, *Indirect transmission of Escherichia coli O157:H7 occurs readily among swine but not among sheep.* Appl Environ Microbiol, 2008. **74**(8): p. 2488-91.

110. Cornick, N.A., *Tylosin and chlorotetracycline decrease the duration of fecal shedding of E. coli O157:H7 by swine.* Vet Microbiol, 2010. **143**(2-4): p. 417-9.

111. Griffin, P.M., et al., *Illnesses associated with Escherichia coli O157:H7 infections. A broad clinical spectrum.* Annals of Internal Medicine, 1988. **109**(9): p. 705-12.

112. Law, D., *Virulence factors of Escherichia coli O157 and other Shiga toxin-producing E. coli.* J Appl Microbiol, 2000. **88**(5): p. 729-45.

113. Bettelheim, K.A., *The non-O157 Shiga-toxigenic (verocytotoxigenic) Escherichia coli; under-rated pathogens.* Crit Rev Microbiol, 2007. **33**(1): p. 67-87.

114. Henderson, H., *Direct and indirect zoonotic transmission of Shiga toxin-producing Escherichia coli.* Journal of the American Veterinary Medical Assocciation, 2008. **232**(6): p. 848-59.

115. CDC. *National Shiga toxin-producing Escherichia coli (STEC) surveillance annual summary, 2009.* 2012 Sep, 10, 2012 January, 2013]; Available from: http://www.cdc.gov/ncezid/dfwed/PDFs/national-stec-surv-summ-2009-508c.pdf.

116. FSIS, *Food Safety and Inspection Service. Shiga toxin-producing Escherichia coli in certain raw beef products.* Federal Register, 2011. **76**: p. 58157-58165.

117. EFSA, *Technical specifications for the monitoring and reporting of verotoxigenic Escherichia coli (VTEC) on animals and food (VTEC surveys on animals and food).* EFSA Journal, 2009. **7**(11): p. 1366.

118. Bielaszewska, M., et al., *Characterisation of the Escherichia coli strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study.* Lancet Infect Dis, 2011. **11**(9): p. 671-6.

CHAPTER 2

Shiga toxin-producing *E. coli* (STEC) in swine: prevalence over the finishing period and characteristics of the STEC isolates

Manuscript based on the data in this chapter has been has been accepted for publication in Epidemiology and Infection on April 10th 2014. (M. Tseng, P. M. Fratamico, L. Bagi, *D. Manzinger*, J. A. Funk**.** Shiga toxin-producing *E. coli* (STEC) in swine: prevalence over the finishing period and characteristics of the STEC isolates)

ABSTRACT

This descriptive longitudinal study was conducted to investigate the fecal shedding of Shiga toxin-producing *E. coli* (STEC) in finishing swine and to characterize the swine STEC isolates that were recovered. Three cohorts of finishing swine (n=50/cohort; total 150 pigs) were included in the longitudinal study. Individual fecal samples were collected every 2 weeks (8 collections/pig) from the beginning (pig age=10 weeks old) to the end (pig age =24 weeks old) of the finishing period. STEC isolates were recovered in at least one sample from 65.3% (98/150) of the pigs, and the frequency distribution of first-time STEC detection during the finishing period resembled an outbreak curve. Nineteen O:H serotypes were identified among the STEC isolates. Most STEC isolates (n=148) belonged to serotype O59:H21 and carried the *stx*$_{2e}$ gene. One O49:H21 STEC isolate carried the *stx*$_{2e}$ and *eae* gene. High prevalence rates of STEC during the finishing period were observed, and STEC isolates in various non-O157 serogroups were recovered. These data enhance understanding of swine STEC epidemiology, and future research is needed to confirm whether or not swine STEC are of public health concern.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) represent a subset of *E. coli* that produce a cytotoxin known as Shiga toxin (Stx) encoded by the *stx* gene (1). Stx has two types (Stx1, Stx2), and each Stx type has multiple variants (Stx1c, Stx1d; Stx2c, Stx2d, Stx2e, Stx2f, Stx2g) (2). STEC infection is associated with outbreaks and sporadic cases of diarrhea and severe clinical diseases in humans, including hemorrhagic colitis (HC) and the potentially fatal hemolytic uremic syndrome (HUS) (2). Therefore, STEC are a critical public health concern, and they cause more than 170000 cases of human illness yearly in the U.S. (3). The infections are most frequently acquired by consuming contaminated food (meat, dairy products, produce, and other foods) and water (2). While STEC serotype O157:H7 is viewed as the serotype associated with most reported outbreaks and severe diseases, non-O157 STEC-associated outbreaks have been increasingly documented (4). Notably, more than 50% of human STEC infections have been attributed to non-O157 STEC (3), but our current knowledge of non-O157 STEC is very limited.

Although cattle are considered the primary STEC reservoirs for human infections, there are food products from other animal species, including pork products, that have been implicated as vehicles in STEC transmission (5-11). A recent STEC outbreak was associated with consuming large cuts of pork from a whole roasted pig (11). Even though investigators were unable to trace back the original sources of STEC in these investigations, there were possible sources of contamination, including intrinsically infected swine or fecal contamination during swine processing, or the STEC could come from non-swine sources, such as cross contamination during preparation, processing or incorporation of contaminated ingredients from other animal

species (8-11). Further research is warranted to elucidate whether swine are a source of STEC contamination. Swine, unlike cattle, may have clinical disease associated with STEC infection (12). Edema disease, often occurring in post-weaning or young finishing age pigs, is caused by STEC strains carrying the *stx*$_{2e}$ gene (12). Cross-sectional epidemiologic studies have been performed to estimate the prevalence of STEC in clinically healthy swine in multiple regions of the world. STEC prevalence in these studies ranged from 0% (13) to 68.3% (14). In the U.S., earlier studies focused on the detection of STEC O157:H7, and the prevalence estimates were low, ranging from 0% (15) to 1.9% (16). In one more recent survey, STEC isolates were recovered in 28.5% of the fecal samples collected, and all of the isolates were categorized as non-O157 serotypes (17). The role that swine play in the epidemiology of STEC, specifically non-O157 STEC, needs further investigation.

Previous studies clearly indicate that pigs can shed STEC, particularly non-O157 STEC serotypes. Yet, they are limited as they employed cross-sectional study designs relying on point estimates of STEC prevalence, and furthermore, only recently have efforts been made to identify and characterize non-O157 serotypes. Little is known about fecal shedding of STEC in clinically healthy swine over time. This longitudinal study was conducted to fill this gap by investigating prevalence of STEC in swine over the finishing period, and to characterize the STEC isolates recovered.

MATERIAL AND METHODS

Study design

This longitudinal study was conducted on two finishing sites (Site A and Site B) within one all-in, all-out multi-site production system in the Midwestern U.S. This means that the pigs originated from the same sow herd, but were reared separately from birth to marketing. These two sites were selected based on convenience and the producers' willingness to participate in the study. Fecal samples were collected from three cohorts of finishing pigs (one cohort on Site A and two cohorts on Site B) from 10 weeks of age until 24 weeks of age (approximately market age). For cohort 1, the farm visits began in May 2011 and ended in August 2011. For cohort 2, the farm visits began in July 2011 and ended in October 2011. For cohort 3, the farm visits began in November 2011 and ended in February 2012.

Site A was a wean-to-finish facility, in which weaned pigs (pig age = 3 to 4 weeks old) were placed into the barns and raised to market age. Site B was a finishing facility, in which pigs, after being housed in a nursery facility (from 3 to 10 weeks of age), were moved into the barns and raised until market age. After a batch of pigs of the same age was placed into the barn, no new pigs were introduced into the barn. These two sites had similar building designs. Each site had two separate buildings, and two separate barns were within each building. Thus, there were four barns on each site. Each barn had 12 pens and was capable of housing a total of 1000 pigs (total site inventory of 4000 pigs). In each barn, there were eight "large" and four "small" pens. Approximately 100 to 125 pigs were placed in each of eight large pens, and 50 pigs were

housed in 2 of the 4 small pens. The remaining small pens were used for housing sick pigs or pigs deemed to be at high risk for disease. Pen dividers allowed pig to pig contact between pens.

Sample collection

A total of 150 individually identified finishing pigs (n=50/cohort; three cohorts) were included in this study. For each cohort, the same proportional sampling scheme was followed. When pigs were 10 weeks of age, 50 pigs in a single barn were randomly selected: six pigs per pen in six large pens, five pigs per pen in two large pens, and two pigs per pen in 2 of the 4 small pens (hospital and at risk pens were empty at the time of placement). The pigs were selected based on random numbers generated by Microsoft Office Excel 2007 (Microsoft, U.S.). From 10 weeks of age, fecal samples were collected from the selected pigs every 2 weeks for a total of eight collections (16 weeks). A total number of 1200 fecal samples (50 pigs/cohort; 8 collections/cohort; 3 cohorts) were planned to be collected. At each collection period, health conditions (e.g. diarrhea, lameness, clinically healthy) of the selected pigs were observed and recorded by research personnel, and health records (e.g. use of medication, signs of clinical symptoms, numbers of pigs that died, potential cause of death) documented by the producers were also recorded.

Individual fecal samples were collected directly from the finishing pigs by gloved hands, and new gloves were used for each pig. Fecal samples were placed into sterile VWR® microbiology/urinalysis specimen containers (VWR International, U.S.). The specimen containers were placed into a cooler under ambient temperature for transportation to the laboratory at Michigan State University in East Lansing, MI. Fecal samples were stored at 2.7ºC

up to 48 h prior to shipping for culture depending on the availability of the shipping service. The fecal samples where then shipped overnight on ice packs to the laboratory located at the Eastern Regional Research Centre of the U.S. Department of Agriculture in Wyndmoor, PA. All fecal samples were processed 24 to 72 h after collection at the farm.

STEC detection and isolation in swine fecal samples

The sample enrichment method was modified from Grant *et al*., 2009 (18). In summary, a five-gram portion of each swine fecal sample was added to 95 mL of tryptic soy broth (TSB) at pH 3.0 in a filter Stomacher bag. The bag was subjected to pummeling in a Stomacher for 30 sec, and then incubated at room temperature for 10 to 15 min. One hundred millilitres of TYTP (TSB + 12.0 g/L yeast extract, 12.5 g/L Trizma base, and 1.0 g/L sodium pyruvate, with a final pH of 8.7) were then added, and samples were incubated without rotation for 15 h at 41˚C.

DNA was then extracted using the PrepSEQ Rapid Spin Sample Preparation Kit (Life Technologies Corporation, U.S.) according to the manufacturer's instructions. A multiplex PCR assay was performed using primers Stx 1/2-F and mod-Stx1/2-R and probes Stx1-P [FAM] and Stx2-P [FAM], targeting *stx*$_1$, *stx*$_2$ and all variants except *stx*$_{2f}$ and Eae-F and mod-Eae-R primers and EaeP [MAXN] probe, targeting the *eae* gene (19), which encodes for the outer membrane protein intimin important for attachment to the intestinal epithelial cells. The PCR assay was performed using 2 µl of template DNA and the TaqMan® Environmental Master Mix 2.0 (Life Technologies Corporation, U.S.) as described by Wasilenko and co-workers (19). The PCR assay was performed in an Applied Biosystems 7500 thermal cycler using a protocol consisting of 95˚C for 10 min, and then 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec.

Enrichment samples that were positive for the *stx* gene were then plated onto CHROMagar STEC (DRG International Inc., U.S.). Although CHROMagar STEC, which contains tellurite, prevents growth of tellurite-sensitve STEC strains, CHROMagar STEC has been suggested to allow growth of 75 to 86.4% of STEC in various serotypes (20-22). From each plate, three presumptive positive colonies were picked and confirmed as STEC using the *stx*$_{1/2}$-*eae* multiplex PCR assay described above. For the confirmed STEC isolates, *stx* gene types (*stx*$_1$, *stx*$_2$) and *stx*$_{2e}$ variant were characterized by PCR assays described in earlier publications (17, 23).

Serotyping of the STEC isolates

At least one confirmed STEC isolate was selected from every positive sample (154 STEC-isolate-positive samples/1040 collected samples) for O:H serotype characterization. For O serogrouping, the confirmed STEC isolates were submitted to the *E. coli* Reference Centre at the Pennsylvania State University in University Park, PA. Antisera were used against serogroups O1 to O181, except for O31, O47, O72, O93, O94, and O122, which are not designated. All STEC isolates with O serogroup information were submitted for H typing at the French Agency for Food, Environmental and Occupational Health and Safety (Maisons-Alfort, France). Selected flagellar antigen genes (H2, H7, H8, H11, H19, H21, H28, H16, H25, H4, H32) were determined as part of a high-throughput real-time PCR system modified from previously published methods (24). The STEC isolates which were not H typeable by the real-time PCR system were submitted to the *E. coli* Reference Centre (University Park, PA) for H typing by PCR-Restriction Fragment Length Polymorphism of the flagellar antigen gene.

Sample size calculation

The sample size was estimated by using the population survey formula in the program Epi Info version 7 (CDC, U.S.). An earlier study by Fratamico *et al*. (17) reported a 28.5% individual animal prevalence of STEC in finishing swine in the U.S. We chose an estimated prevalence of 30.0% with a 10.0% confidence limit and a population size of 1000, and the confidence was set as 90.0%. This resulted in a target sample size of 50 for each cohort to estimate prevalence at each sampling period.

Variables and statistical methods

All data were recorded and managed in Microsoft Office Excel 2007 (Microsoft, U.S.). A pig was considered STEC positive when at least one confirmed STEC isolate was recovered from the fecal sample. The outcome of interest was the proportion (prevalence rate) of pigs positive for STEC at each farm visit. After data collection was completed, data validation was performed by comparing the records of farm visits and STEC isolation results from the laboratory to the database. Descriptive statistics of the proportion of pigs positive for STEC at each farm visit was performed in Microsoft Office Excel 2007 (Microsoft, U.S.).

For each pig positive for STEC, the duration of STEC shedding was defined as the time interval (days) between the first and last sampling date of positive STEC isolation, which was before three consecutive STEC negative samples. An additional 14 days was counted for each positive sampling date to account for sampling intervals. Right censored data included pigs that died or were shipped to market before the observation of three consecutive STEC negative

samples. Survival analysis using the Kaplan-Meier method was performed to analyse the duration of STEC shedding in finishing swine by STATA 13 (STATACorpLP, U.S.). The equality of survivor curves by cohort, gender, and STEC serotype was examined by the Peto-Peto-Prentice test. P-values <0.05 were considered significant.

RESULTS

Finishing pigs and sample collection

A total of 150 pigs (50 pigs in each cohort, three cohorts in total) were included in the longitudinal sampling. Ten (6.7%) out of the 150 pigs died during the study period. Diarrhea was observed in at least collection period in 12.0% (6/50) of the pigs in cohort 1, 12.0% (6/50) of the pigs in cohort 2, and 10.0% (5/50) of the pigs in cohort 3, and samples were collected from these pigs with diarrhea. There were seven instead of eight farm visits for cohort 1 due to marketing of the pigs prior to the final collection, and eight farm visits occurred for cohort 2 and for cohort 3. Eight farm visits were completed in 43.3% (65/150) of the pigs, and seven farm visits were completed in 36.7% (55/150) of the pigs. At the end of sample collection, a total of 1040 fecal samples were collected. The main reasons why samples may not have been collected at farm visits were that the pigs were too ill, the pigs were dead, or the pigs were shipped for marketing before sample collection (n=14 in cohort 1, n=0 in cohort 2, n=9 in cohort 3). The sample collection time and demographic information of the finishing pigs are summarized in Table 2.1.

Distribution of STEC positive pigs over the finishing period

STEC isolates were recovered from at least one sample from 65.3% (98/150) of the pigs. Specifically, STEC isolates were recovered in a least one sample from 62.0% (31/50) of the pigs in cohort 1, 54.0% (27/50) of the pigs in cohort 2, and 80.0% (40/50) of the pigs in cohort 3.

Figure 2.1.a illustrates the proportions (prevalence rates) of pigs with STEC isolates by pig age in weeks over the finishing period, and Figure 2.1.b displays numbers of pigs at the age of first-time STEC detection. The highest prevalence rates of STEC positive pigs were detected when pigs were 18 weeks old in cohort 1 (47.9%), 16 weeks old in cohort 2 (39.5%), and 14 weeks old in cohort 3 (59.2%). The numbers of pigs at the age of first-time STEC detection peaked at the same age as the proportions of STEC positive pigs in every cohort. At the end of finishing period, the prevalence rates of STEC positive pigs were lower: 6.3% in cohort 1, 0% in cohort 2, and 6.7% in cohort 3. STEC isolates were recovered in one fecal sample in 36.7% (55/150), two samples in 20.7% (31/150), three samples in 7.3% (11/150), and four samples in 0.7% (1/150) of the pigs. No STEC isolates were ever recovered from samples of the pigs that had diarrhea.

Characterization of the STEC isolates

Among the 1040 swine fecal samples, total 285 STEC isolates were recovered from 83.2% (154/185) *stx*-gene-positive samples. The presence of virulence genes (*stx*$_1$, *stx*$_2$, *stx*$_{2e}$, *eae*) were determined by PCR in all the 285 STEC isolates. Most of the STEC isolates (97.9%, 279/285) carried the *stx*$_{2e}$ gene. Four STEC isolates carried the *stx*$_1$ gene. Two STEC isolates carried the *stx*$_2$ gene and were negative for *stx*$_{2e}$. The *eae* gene was detected in only one of the 285 STEC isolates.

For O:H serotype characterization, at least one isolate was selected from every STEC positive pig at each farm visit. A total of 200 STEC isolates were submitted for O:H serotype determination. Among the 200 STEC isolates, ten different O serogroups were identified while some isolates (n=29) were O serogroup non-typeable. Nine H types were identified in the 200

STEC isolates, and together, nineteen different O:H serotypes were identified. A majority (73.6%, 148/201) of the STEC isolates were categorized as serotype O59:H21. The results of O:H serotype and virulence gene (*stx*$_1$, *stx*$_2$, *stx*$_{2e}$, *eae*) characterization are summarized in Table 2.2.

Estimation of duration of STEC shedding in finishing swine

The pigs (n=19) with samples positive for STEC isolates belonging to more than one serotype were excluded from the survival analysis. Therefore, data from 79 pigs with samples positive for STEC isolates for one serotype were included in the survival analysis. In these 79 pigs, data from 34.2% (27/79) of the pigs were right-censored. In the 27 right-censored pigs, 7.4% (2/27) of the pigs died during the study period, and 40.7% of them (11/27) were shipped to market before the study ended. The event (three consecutive STEC negative samples) was not observed in the remaining 14 of the 27 censored pigs before the study ended. In the 79 pigs included in the survival analysis, the median of the duration of STEC fecal shedding was 28 days. Kaplan-Meier analysis suggested that the cumulative probability of finishing swine shedding STEC for 28 days was 30.0%. The shortest shedding duration was 14 days at a probability of 53.2%. The longest shedding duration was 56 days at a probability of 6.7%. Figure 2.2. illustrates the Kaplan-Meier survival curve of duration of fecal STEC shedding in finishing swine. No significant differences were observed among survival curves by cohorts (p= 0.20), gender (p=0.96), and STEC serotypes (p=0.84).

DISCUSSION

This study represents the first longitudinal study of STEC shedding in commercial swine. Our results indicated that finishing swine shed STEC at relatively high prevalence rates during the finishing period. Previous studies have reported a wide range of STEC prevalence in swine, but most of them were cross-sectional study designs, challenging direct comparison to this study (13-15, 17). In addition to study designs, comparison is limited because of the type of sample collected, sample collection method, and STEC isolation protocols, which vary widely across these previous reports. Although only one production company participated in this study, this company sufficiently represents the majority of conventional swine production systems in the U.S. (all-in, all-out multi-site production, etc.)

Variations in STEC prevalence rates over time were observed in this study, and this highlights the importance of longitudinal sampling to determine the STEC shedding status on farm. A shape similar to an outbreak curve was observed when plotting the numbers of pigs at the age of first-time STEC detection, and the outbreak-like curves were observed from the results of all three of the cohorts. This may suggest that the pigs were exposed to a single source of STEC infection in the beginning of finishing period. The STEC prevalence rates were different by pig age in these three cohorts. At the cohort level, no significant change of barn environments and farm management protocols was observed during the study period in these three cohorts. At the individual pig level, no significant factors (e.g. sex and health condition) were observed in the STEC-positive pigs. Common sources of STEC infection may be from the finishing barn environment, the finishing diet, or other factors. To further determine whether the STEC strains were from a common source of infection, it will be essential for future studies to

examine the genetic relatedness of the swine STEC strains by molecular genotyping methods. Moreover, expanding the scope of observations to more finishing sites will be crucial to understand the potential cohort-level or individual risk factors associated with the dynamics of STEC prevalence on swine farms.

The duration of detectable shedding in this study was similar to that described in experimentally-challenged swine. Regardless of the inoculation dose, STEC O157:H7 strains were recovered in fecal samples 14 to16 days after inoculation (25). However, when inoculated with a higher dose at 10^{10} CFU/strain/animal, STEC O157:H7 strains were able to be recovered in fecal samples 58 to 60 days after inoculation (25). Together, these data suggest that some pigs in this study might be exposed to a higher dose of STEC during the finishing period, and this resulted in a longer duration of STEC shedding. Moreover, re-infection of STEC from other pigs in the same barn may occur. Re-infection of STEC from pigs in neighboring barns may also occur because of potential pig to pig contact through the pen dividers in the barns in this study. Additionally, the differences of shedding duration may be attributed to differential colonization ability of different STEC strains and the host itself. One study reported that no difference in duration of STEC shedding comparing pigs housed on crates and on cement floors (26). However, what the potential factors are and how these factors contribute to the duration of STEC shedding in pigs remain largely unknown. A better understanding of the factors associated with the duration of shedding will be helpful for strategizing future plans for controlling STEC shedding on farms.

The STEC isolates recovered in this study belonged to a number of different serotypes. However, none belonged to O-serogroups O138, O139, O141, and O147, which are more frequently associated with edema disease in swine (12). The serotypes were different from those

reported in the swine STEC isolates from the NAHMS 2000 study (17). Different STEC isolation methods may contribute to the observations of different serotypes. For example, CHROMagar STEC, which was used in this study, contains tellurite, and some STEC strains were not able to grow on this medium (20-22). Nevertheless, CHROMagar STEC was suggested to be useful for STEC isolates recovery from fecal samples, and it allowed the growth of 75% to 86.5% STEC strains in a wide variety of serotypes that were examined in these previous study (20-22).

The majority of the STEC isolated in the current study were serotype O59:H21. This particular serotype has not yet been reported in human cases but was reported in STEC isolates from food (beef, pork, and others) (27). Some serotypes and O-serogroups of the swine STEC isolated in this study have been reported in human cases. For instance, STEC O59:H19 (28), O20 (29), O49, O98, and O119 (4) have been associated with human cases. Results of this study show that swine carry a variety of different STEC serotypes, and this was observed with sampling only three groups of pigs in one swine production company.

Most swine STEC isolates in this study carried the stx_{2e} gene. Interestingly, Stx2e-producing *E. coli* strains have not only been recovered in pigs, but also in humans with HUS (30) and uncomplicated diarrhea (31-34). Although no source of infection was ascertained in these human cases, the association between Stx2e-producing *E. coli* and human illness requires further examination. The *eae* gene was detected in only one STEC isolate (O49:H21, carrying the stx_{2e} gene) in this study. The presence of the *eae* gene in swine STEC was not consistent in previous studies. Some studies reported that the *eae* gene was not detected in swine STEC (35), while others have reported detection of *eae* in STEC O157:H7 strains recovered from swine colon fecal samples and carcass swabs at slaughter houses (36). Although it is well-known that intimin is

essential in STEC attachment in humans (37), it has been suggested that intimin is not required for STEC O157:H7 colonization in pigs (38). This may partially explain the fact that only one out of 285 STEC isolates in this study was *eae*-positive. However, outbreaks and cases associated with *eae*-negative STEC strains were reported (39). Many novel adhesins have been proposed as possibly being important in *eae*-negative STEC, such as the STEC autoagglutinating adhesin encoded by the *saa* gene (40). To better assess the potential risks posed by swine STEC, there is a need to more extensively examine the virulence gene profiles in swine STEC isolates.

Swine have not been viewed as an important STEC reservoir. However, the findings of this study provide insights into the epidemiology of fecal STEC shedding in finishing swine. It was observed that swine can shed STEC at high prevalence rates during the finishing period. The swine STEC isolates recovered in this study belonged to various non-O157 STEC serotypes, and *E. coli* O157:H7 was not isolated. There is increasing awareness of the public health burden associated with non-O157 STEC infections (3). In addition, the recent emergence of highly virulent non-O157 STEC possessing unusual virulence gene combinations stress the need to further understand these pathogens (39). From this study, relative high prevalence rates of STEC isolation was observed during swine finishing period. Moreover, STEC isolates in various non-O157 serotypes have been recovered. Future study is warranted to confirm whether or not swine are an important source of human STEC infections, specifically non-O157 STEC.

ACKNOWLEDGEMENTS

This work was supported by the National Pork Board (project number 12-069). Sincere thanks to the producers who participated in this research, MSU students: Dr. Alda Pires, Alexandra Buckley, Allan Mergener, Tasha Likavec, Joel Sparks for assistance with sample collection and shipment. Dr. Chitrita DebRoy and the *E. coli* Reference Centre at The Pennsylvania State University, Dr. Patrick Fach and Dr. Sabine Delannoy at the French Agency for Food, Environmental and Occupational Health and Safety are acknowledged for assistance with isolate characterization.

APPENDIX

Table 2.1. Sample time periods, demographic information and numbers of fecal samples of finishing pigs

	Cohort 1	Cohort 2	Cohort 3
Production site	A	B	B
Sample collection period	31 May 2011 (visit 1) - 22 August 2011 (visit 7)	18 July 2011 (visit 1) - 22 October 2011 (visit 8)	21 November 2011 (visit 1) - 18 February 2012 (visit 8)
Number of animals in each cohort	50	50	50
Number of animals died during study period	2	6	2
Sex			
male	23	17	24
female	27	33	26
Number of fecal samples collected	320	357	363

Table 2.2. Distribution of STEC isolates by O:H serotype, Shiga toxin gene subtypes, and *eae*

		Number of isolates positive for			
Serotype	Total number of isolates	stx_{2e}	other stx_2 variants	stx_1 variants	*eae*
O15:H45	1	1	0	0	0
O15:H+[e]	1	0	1	0	0
O20:H21	1	1	0	0	0
O49:H21	1	1	0	0	1
O59:H19	3	3	0	0	0
O59:H21	148	148	0	0	0
O59:H21/H4[b]	3	3	0	0	0
O59:H19/H21[c]	2	2	0	0	0
O59/O54[d]:H21	2	2	0	0	0
O89:H19	1	1	0	0	0
O98:H12	4	0	0	4	0
O98:H19	1	1	0	0	0
O115:H19	1	1	0	0	0
O119:H21	1	1	0	0	0
O167:H21	1	1	0	0	0
ONT[e]:H19	19	19	0	0	0
ONT[e]:H4	7	7	0	0	0

Table 2.2. (cont'd)

Serotype	Total number of isolates	Number of isolates positive for			
		stx$_{2e}$	other *stx*$_2$ variants	*stx*$_1$ variants	*eae*
ONT[e]:H21	2	2	0	0	0
ONT[e]:H27	1	0	1	0	0
Total	200	194	2	4	1

[a]H+, flagellar gene *fli*C present, there were two bands for PCR.

[b]These isolates were positive for H21 and H4 genes by part of the high-throughput real-time PCR platform.

[c]These isolates were positive for H19 and H21 genes by part of high-throughput real-time PCR platform.

[d]These isolates were positive with both O59 and O54 antisera.

[e]ONT, O antigen non-typeable.

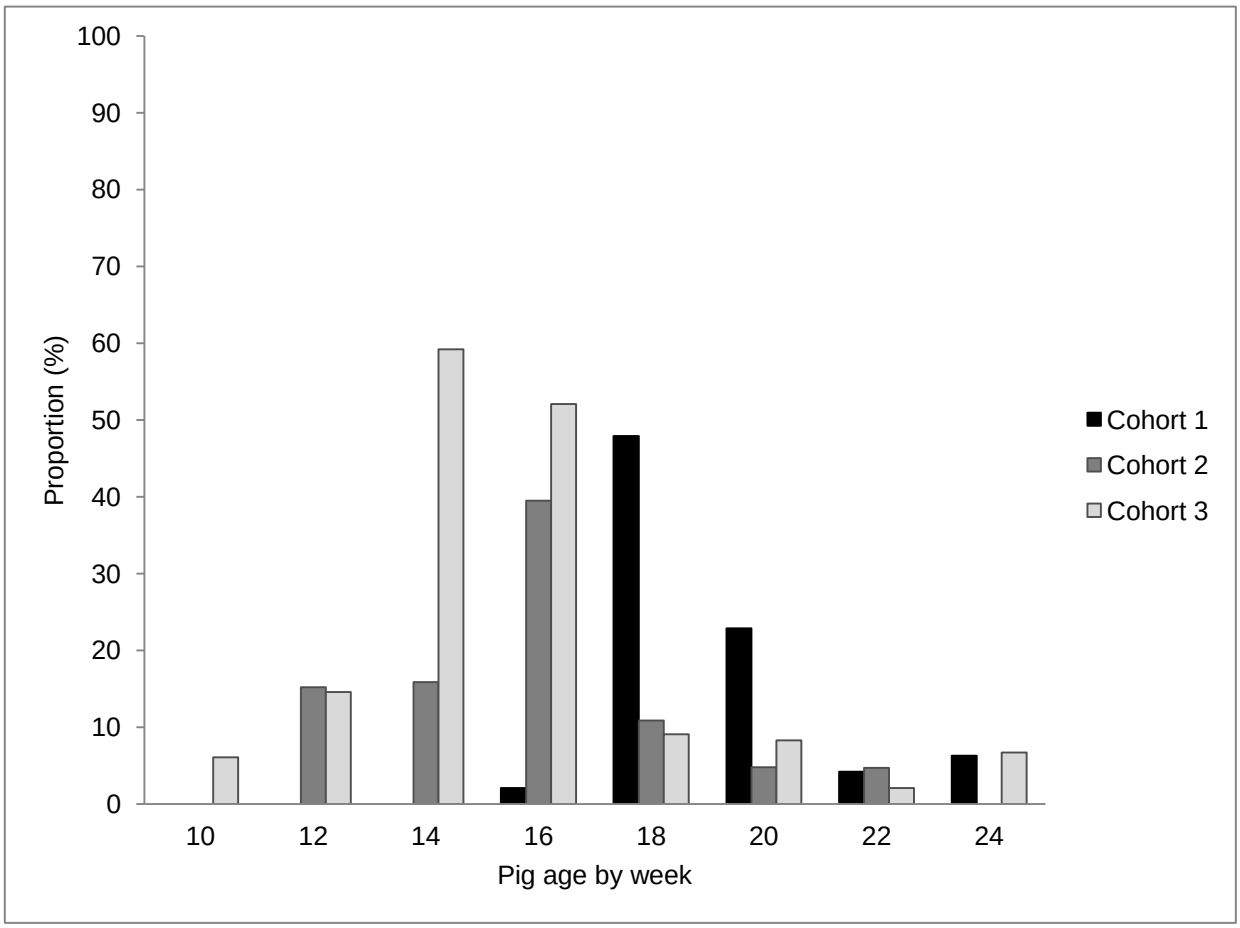


Figure 2.1.a. Proportion of pigs from which STEC were isolated by pig age over the finishing period

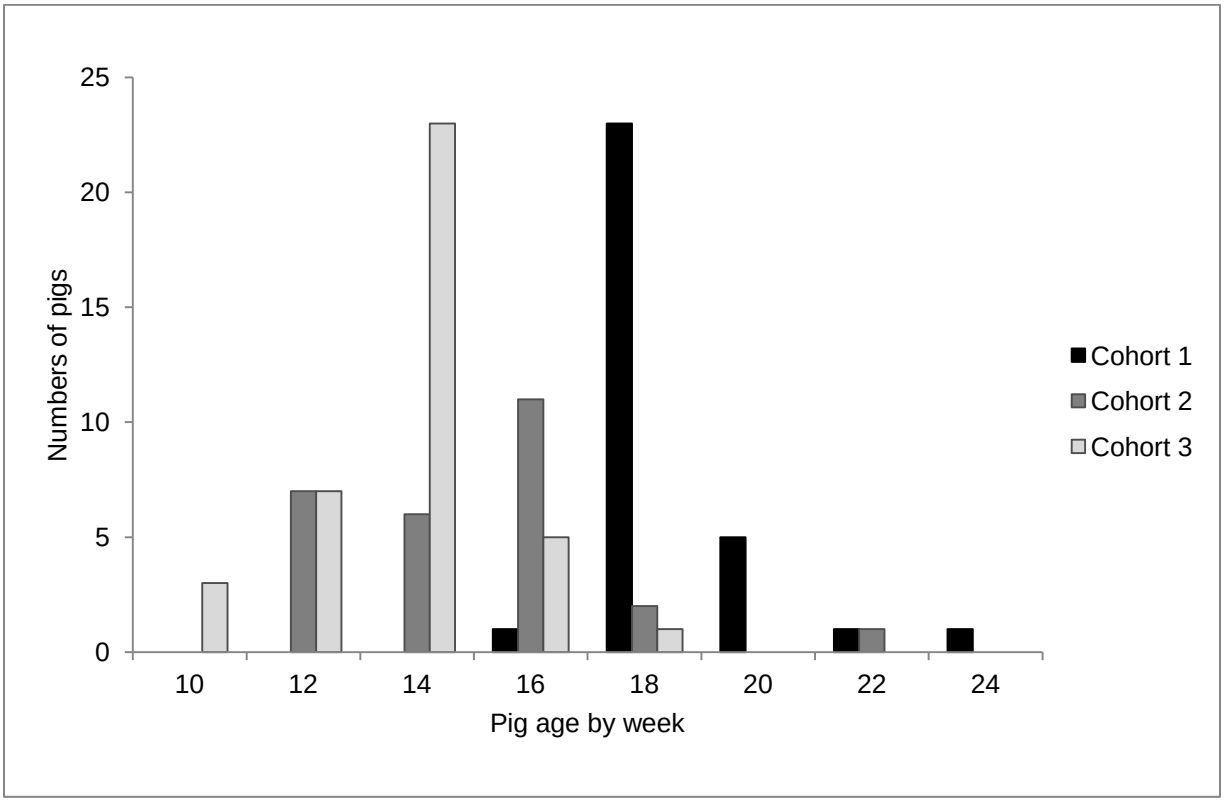


Figure 2.1.b. Frequency distribution of pigs at the age of first-time STEC isolation

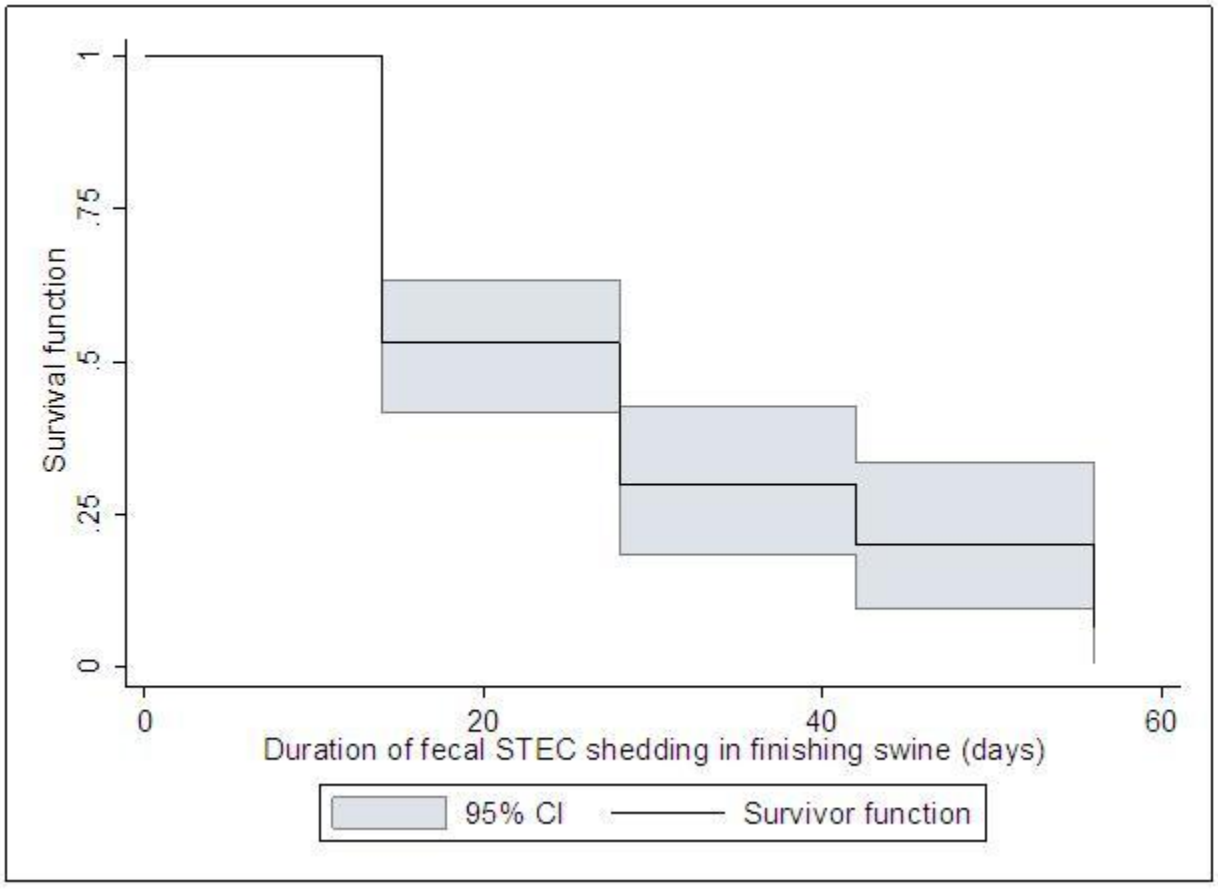


Figure 2.2. Kaplan-Meier survival curves for duration of fecal STEC shedding in finishing swine

REFERENCES

REFERENCES

1. O'Brien, A.D. and G.D. LaVeck, *Purification and characterization of a Shigella dysenteriae 1-like toxin produced by Escherichia coli.* Infection and Immunity, 1983. **40**(2): p. 675-83.

2. Gyles, C.L., *Shiga toxin-producing Escherichia coli: an overview.* J Anim Sci, 2007. **85**(13 Suppl): p. E45-62.

3. Scallan, E., et al., *Foodborne Illness Acquired in the United States—Major Pathogens.* Emerging Infectious Diseases, 2011. **17**(1): p. 7-15.

4. CDC. *National Shiga toxin-producing Escherichia coli (STEC) surveillance annual summary, 2009.* 2012 Sep, 10, 2012 January, 2013]; Available from: http://www.cdc.gov/ncezid/dfwed/PDFs/national-stec-surv-summ-2009-508c.pdf.

5. CDC, *Escherichia coli O157:H7 outbreak linked to commercially distributed dry-cured salami- Washington and California, 1994.* Morbidity and Mortality Weekly Report, 1995. **44**: p. 157-160.

6. CDC, *Community outbreak of hemolytic uremic syndrome attributable to Escherichia coli O111:NM-South Australia, 1995.* Morbidity and Mortality Weekly Report, 1995. **44**: p. 550-558.

7. Paton, A.W., et al., *Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing Escherichia coli.* J Clin Microbiol, 1996. **34**(7): p. 1622-7.

8. Williams, R.C., et al., *Illness outbreak associated with Escherichia coli O157:H7 in Genoa salami. E. coli O157:H7 Working Group.* Canadian Medical Association Journal, 2000. **162**(10): p. 1409-13.

9. MacDonald, D.M., et al., *Escherichia coli O157:H7 outbreak linked to salami, British Columbia, Canada, 1999.* Epidemiol Infect, 2004. **132**(2): p. 283-9.

10. Conedera, G., et al., *A family outbreak of Escherichia coli O157 haemorrhagic colitis caused by pork meat salami.* Epidemiol Infect, 2007. **135**(2): p. 311-4.

11. Trotz-Williams, L.A., et al., *Pork Implicated in a Shiga Toxin-producing Escherichia coli O157:H7 Outbreak in Ontario, Canada.* Canadian Journal of Public Health. Revue Canadienne de Sante Publique, 2012. **103**(5): p. e322-6.

12. Fairbrother, J.M., Gyles CL, *Colibacillosis*, in *Diseases of Swine*, L.K. Jeffrey Zimmerman, Alejandro Ramirez, Kent Schwartz,Gregory Stevenson Editor. 2012, John Wiley and sons, Inc.: West Sussex, UK. p. 723-747.

13. Pinaka, O., et al., *Shiga toxin-producing Escherichia coli in Central Greece: prevalence and virulence genes of O157:H7 and non-O157 in animal feces, vegetables, and humans.* European Journal of Clinical Microbiology and Infectious Diseases, 2013.

14. Borie, C., et al., *Prevalence and characterization of enterohaemorrhagic Escherichia coli isoated from healthy cattle and pigs slaughtered in Santiago, Chile.* Archivos De Medicina Veterinaria, 1997. **29**(2): p. 205-212.

15. Bush, E., *U.S. swine herd appears free of Escherichia coli O157:H7.* Food Safety Digest, 1997. **4**.

16. Feder, I., et al., *Isolation of Escherichia coli O157:H7 from intact colon fecal samples of swine.* Emerg Infect Dis, 2003. **9**(3): p. 380-3.

17. Fratamico, P.M., et al., *Prevalence and characterization of Shiga toxin-producing Escherichia coli in swine feces recovered in the National Animal Health Monitoring System's Swine 2000 study.* Appl Environ Microbiol, 2004. **70**(12): p. 7173-8.

18. Grant, M.A., M.A. Mogler, and D.L. Harris, *Comparison of enrichment procedures for shiga toxin-producing Escherichia coli in wastes from commercial swine farms.* J Food Prot, 2009. **72**(9): p. 1982-6.

19. Wasilenko, J.L., et al., *Influence of primer sequences and DNA extraction method on detection of non-O157 Shiga toxin-producing Escherichia coli in ground beef by real-time PCR targeting the eae, stx, and serogroup-specific genes.* Journal of Food Protection, 2012. **75**(11): p. 1939-50.

20. Hirvonen, J.J., A. Siitonen, and S.S. Kaukoranta, *Usability and performance of CHROMagar STEC medium in detection of Shiga toxin-producing Escherichia coli strains.* Journal of Clinical Microbiology, 2012. **50**(11): p. 3586-90.

21. Wylie, J.L., et al., *Evaluation of a new chromogenic agar medium for detection of Shiga toxin-producing Escherichia coli (STEC) and relative prevalences of O157 and non-O157 STEC in Manitoba, Canada.* Journal of Clinical Microbiology, 2013. **51**(2): p. 466-71.

22. Gouali, M., et al., *Evaluation of CHROMagar STEC and STEC O104 chromogenic agar media for detection of Shiga Toxin-producing Escherichia coli in stool specimens.* Journal of Clinical Microbiology, 2013. **51**(3): p. 894-900.

23. Blanco, M., et al., *Genes coding for enterotoxins and verotoxins in porcine Escherichia coli strains belonging to different O:K:H serotypes: relationship with toxic phenotypes.* Journal of Clinical Microbiology, 1997. **35**(11): p. 2958-63.

24. Bugarel, M., et al., *Virulence gene profiling of enterohemorrhagic (EHEC) and enteropathogenic (EPEC) Escherichia coli strains: a basis for molecular risk assessment of typical and atypical EPEC strains.* BMC Microbiol, 2011. **11**: p. 142.

25. Booher, S.L., N.A. Cornick, and H.W. Moon, *Persistence of Escherichia coli O157:H7 in experimentally infected swine.* Vet Microbiol, 2002. **89**(1): p. 69-81.

26. Cornick, N.A. and A.F. Helgerson, *Transmission and infectious dose of Escherichia coli O157:H7 in swine.* Appl Environ Microbiol, 2004. **70**(9): p. 5331-5.

27. Beutin, L., et al., *Identification of human-pathogenic strains of Shiga toxin-producing Escherichia coli from food by a combination of serotyping and molecular typing of Shiga toxin genes.* Appl Environ Microbiol, 2007. **73**(15): p. 4769-75.

28. Galli, L., et al., *Virulence profile comparison between LEE-negative Shiga toxin-producing Escherichia coli (STEC) strains isolated from cattle and humans.* Veterinary Microbiology, 2010. **143**(2-4): p. 307-13.

29. Kappeli, U., et al., *Human infections with non-O157 Shiga toxin-producing Escherichia coli, Switzerland, 2000-2009.* Emerg Infect Dis, 2011. **17**(2): p. 180-5.

30. Thomas, A., et al., *Isolation of Vero cytotoxin-producing Escherichia coli serotypes O9ab:H- and O101:H-carrying VT2 variant gene sequences from a patient with haemolytic uraemic syndrome.* Eur J Clin Microbiol Infect Dis, 1994. **13**(12): p. 1074-6.

31. Pierard, D., et al., *Diarrhoea associated with Escherichia coli producing porcine oedema disease verotoxin.* Lancet, 1991. **338**(8769): p. 762.

32. Muniesa, M., et al., *Characterization of a Shiga toxin 2e-converting bacteriophage from an Escherichia coli strain of human origin.* Infect Immun, 2000. **68**(9): p. 4850-5.

33. Friedrich, A.W., et al., *Escherichia coli harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms.* J Infect Dis, 2002. **185**(1): p. 74-84.

34. Beutin, L., et al., *Characterization of Shiga toxin-producing Escherichia coli strains isolated from human patients in Germany over a 3-year period.* J Clin Microbiol, 2004. **42**(3): p. 1099-108.

35. Fratamico, P.M., et al., *Characterization of Shiga toxin-producing Escherichia coli strains isolated from swine feces.* Foodborne Pathogen and Disease, 2008. **5**(6): p. 827-38.

36. Lenahan, M., et al., *Molecular characterization of IrishE. coliO157:H7 isolates of human, bovine, ovine and porcine origin.* Journal of Applied Microbiology, 2009. **107**(4): p. 1340-1349.

37. Frankel, G. and A.D. Phillips, *Attaching effacing Escherichia coli and paradigms of Tir-triggered actin polymerization: getting off the pedestal.* Cell Microbiol, 2008. **10**(3): p. 549-56.

38. Jordan, D.M., S.L. Booher, and H.W. Moon, *Escherichia coli O157:H7 does not require intimin to persist in pigs.* Infect Immun, 2005. **73**(3): p. 1865-7.

39. Bielaszewska, M., et al., *Characterisation of the Escherichia coli strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study.* Lancet Infect Dis, 2011. **11**(9): p. 671-6.

40. Paton, A.W., et al., *Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic Escherichia coli strains that are virulent for humans.* Infect Immun, 2001. **69**(11): p. 6999-7009.

CHAPTER 3

Molecular Characterization of Shiga Toxin-Producing *E. coli* (STEC) from Finishing Swine in a Longitudinal Study

This manuscript is in preparation for submission to the Journal of Applied and Environmental Microbiology.

ABSTRACT

Shiga toxin-producing *E. coli* (STEC) infections are a critical public health concern because of the severe clinical outcomes in humans, for example, potentially fatal hemolytic uremic syndrome. Determining the presence or absence of virulence genes is essential in assessing the potential pathogenecity of STEC strains. Currently, there is limited information about the virulence genes carried by swine STEC strains. This study was conducted to examine the presence and absence of a large panel of virulence genes in STEC strains recovered from finishing swine in a longitudinal study. A subset of STEC strains was analyzed by pulsed field gel electrophoresis (PFGE) to examine their genetic relatedness. Swine STEC strains (n=155) were analyzed by a high-throughput real-time PCR array system, which included 69 virulence gene targets. Seventeen different combinations of virulence gene profiles and serotypes were determined in the swine STEC strains. The majority of the swine STEC strains (n=120) belonged to serotype O59:H21, and carried the same virulence gene profile. The *eae* gene and two *nle* variants were detected in one swine STEC strain (O49:H21). Other genes encoding adhesins were identified, for example, the *iha* gene (n=154). The PFGE results revealed that swine STEC strains from pigs raised in the same finishing barn were clonally related. This work is the critical first step to understand the potential pathogenic mechanisms of swine STEC in human disease.

INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) infections are a critical public health concern, leading to 170,000 cases of human illness (1) and an economic burden of 280 million dollars (2) annually in the United States. STEC represent a subset of *E. coli* that produce a cytotoxin known as Shiga toxin, encoded by a phage carrying the *stx* gene (3, 4). The term enterohemorrhagic *E. coli* (EHEC) was often used to define a subgroup of STEC strains, which are associated with outbreaks and severe clinical cases (5). STEC infections are often acquired from consuming contaminated food or water (6), and cattle are viewed as the most important animal reservoirs for STEC (7). Nevertheless, many other food products, including pork products, have been confirmed as vehicles of STEC transmission in a number of outbreaks (8-14).

Although the source of contamination of the pork products was uncertain in these outbreaks (8-14) , the likelihood that on-farm pigs are the source of STEC contamination cannot be overlooked. Unlike cattle which do not usually present clinical symptoms under STEC infections, pigs, specifically post-weaning and young finishing pigs, suffer from edema disease caused by STEC strains carrying the stx_{2e} variant gene (15). Epidemiological studies conducted in different regions of the world have reported wide ranging prevalences of STEC in swine populations (16-18). However, the epidemiology and characteristics of STEC carried by on-farm pigs and whether swine STEC strains contribute to human disease burden remain largely unknown.

Human STEC infections are associated with a range of clinical presentations: diarrhea, hemorrhagic colitis (HC), and the life-threatening hemolytic uremic syndrome (HUS) (5, 19). The pathogenesis of STEC in human patients has been reviewed elsewhere (7, 20-24). Although

Shiga toxins are known to be crucial in STEC pathogenesis, as they inhibit host cell protein synthesis (25) and are involved in host cell apoptosis (reviewed in (26)), virulence factors other than Shiga toxins are associated with the capability of STEC strains to cause disease (27). Some fimbriae are proposed to be involved in the initial attachment steps of STEC on host colonocytes, such as the long polar fimbriae encoded by the *lpf* gene, which has many variants (21, 28). Following the initial attachment, some STEC strains express intimin, encoded by *eae* gene, which is located on the pathogenicity island locus of enterocyte effacement (LEE). Intimin contributes to the formation of intimate attachment to host cells (29). For those STEC strains, which cause HC and HUS in human patients but do not express intimin (LEE-negative STEC), various potential adhesins have been identified, for example, the STEC autoagglutinating adhesin encoded by the *saa* gene (30). A number of other virulence factors other than Stx have been proposed to contribute to STEC pathogenesis, for instance, catalase peroxidase encoded by *kat*P gene (31). Moreover, some non-LEE encoded effector (*nle*) genes are associated with STEC strains isolated from human patients with severe clinical diseases, namely HC or HUS (27, 32).

Knowing that various combinations of virulence factors contribute to STEC pathogenesis, it is essential to determine the presence or absence of specific virulence genes to better assess the potential pathogenicity of STEC strains (27, 32, 33). A few studies have examined the virulence gene profiles of swine STEC strains (34-38). However, every study selected different panels of virulence genes, and this fact makes it highly challenging to compare results across different studies. The presence of many virulence genes, for example the *nle* genes and their variants, has not been determined in swine STEC strains. In general, there is a significant knowledge gap about virulence genes carried by swine STEC strains. To fill in the current knowledge gap and better evaluate the potential pathogenicity of swine STEC strains,

this study was conducted to examine the presence of a large panel of virulence genes in STEC strains recovered from finishing pigs in a longitudinal study. Moreover, the genetic relatedness of these swine STEC strains was also examined to understand the transmission dynamics of STEC in finishing swine.

MATERIALS AND METHODS

Swine STEC strains

A total of 155 STEC strains recovered from 97 finishing pigs in a longitudinal study were included in this study. In the longitudinal study, individual fecal samples were collected from three cohorts of finishing pigs (n=50/cohort, n=150 in total). Each cohort was raised in separated finishing barns at two finishing sites (cohort 1 at site A, cohorts 2 and 3 at site B). The samples were collected every two weeks during the finishing period in each cohort (8 farm visits/cohort). A sample was considered STEC positive when an STEC isolate was recovered. The presence of virulence genes (*stx*$_1$, *stx*$_2$, *stx*$_{2e}$, *eae*) and O:H serotype were characterized in these STEC isolates previously (M. Tseng, P. M. Fratamico, L. Bagi, D. *Manzinger*, and J. A. Funk, in press). At least one STEC strain was selected from each positive sample for virulence gene characterization. STEC strains in different O serogroups from the same sample were also included in the study.

Selection of virulence gene targets

The general rationale to select virulence gene targets was based on their functions, roles in pathogenesis, association with human illness and/or disease severity in human patients. According to the previous characterization results, only one among the 155 swine STEC strains carried the *eae* gene (M. Tseng, P. M. Fratamico, L. Bagi, D. *Manzinger*, and J. A. Funk, in press). Additionally, all swine STEC strains recovered in the longitudinal study were in non-

O157 serogroups. Therefore, we expanded the list of virulence gene targets to include putative genes encoding adhesins, toxins, fimbriae, and others in LEE-negative and non-O157 STEC strains. We also included virulence genes associated with swine edema disease. The complete list of the virulence genes (n=69) is summarized in Table 3.1.

High-throughput real-time PCR microarray

The BioMark™ real-time PCR system (Fluidigm, USA) was used for high-throughput microfluidic real-time PCR amplification using 96.96 dynamic arrays (Fluidigm). Amplifications were performed using the EvaGreen DNA binding dye (Biotium Inc., Hayward, CA) with Gene expression master mix in accordance with the recommendations of the manufacturer (Applied Biosystems, Courtaboeuf, France). The thermal profile comprised 10 min at 95°C, followed by 35 cycles of 95°C for 15s and 60°C for 1 min, followed by a melting curve analysis.

Besides the 69 selected virulence genes, O-group associated genes O26, O157, O145, O103, O111, O121, O45, O118, O128, O146, O91, O104, O113, and O55 were included in the PCR microarray. Moreover, the microarray chip also contained gene targets for flagellar genes H11, H7, H21, H2, H28, H8, H19, H16, H25, H4, and H32.

Strain selection strategy for pulsed-field gel electrophoresis (PFGE)

A subset of swine STEC strains (n=56) was selected for PFGE analysis to determine their genetic relatedness. The selection criteria were based on serotype, virulence profiles and epidemiologic information of the pigs. Within the predominant serotype O59:H21, we selected

strains recovered in the early, middle, and late stage of the finishing period. We also included STEC strains in the same serotype and recovered from the same pig at different farm visits over the finishing period. STEC strains in the same serotype but with different virulence gene profiles were selected for PFGE analysis. In total, 29 O59:H21 STEC strains were selected, and two O59:19 STEC strains were selected. Additionally 13 STEC strains in serotype O untypeable:H19, four O98:H12 STEC strains, and one O98:H19 strain were also included in the PFGE analysis.

PFGE

PFGE was conducted according to the standardized Centers of Disease Control and Prevention (CDC) PulseNet protocol (39). In summary, STEC DNA was embedded in agarose and digested with 50 U of XbaI for 2h at 37 ºC. A CHEF DR-III system (BioRad, Munich, Germany) was used to separate the restriction fragments by electrophoresis at pulse times of 2.16-54.17 s in 0.5x Tris-borate-EDTA buffer with 50 μM thiourea at 14 ºC for 16.2h. The H9812 *Salmonella enterica* serovar Braenderup strain (CDC, Atlanta, GA) was utilized as a molecular size marker. BioNumerics software package 6.6 (Applied Maths, Ghent, Belgium) was used to analyze the PFGE restriction-digested band patterns. The dendrogram was built by analyzing Dice coefficients and the UPGMA method with 0.5% band position tolerance. The genetic relatedness of the strains was assessed by the percentage similarity between the PFGE patterns.

RESULTS

Virulence gene profiles of swine STEC strains

The frequency distribution of the 155 swine STEC strains by their serotype and virulence gene profiles is summarized in Table 3.2. There were 12 virulence gene profiles within our swine STEC strains. In combination with the serotypes, there were 17 different STEC strains by combinations of serotypes and virulence gene profiles. Most strains (80.6%, 125/155) carried the same virulence gene profile (virulence gene profile 1), which contained *stx*$_{2e}$, *iha*, ecs1763, *lpfA*-O113, *estIa* (*STa*), *ehaA*, *paa*, *terE*, and *ureD*. The second most prevalent virulence gene profile, virulence gene profile 3, was found in 9.6% of the strains (15/155). This virulence gene profile 3 contained *stx*$_{2e}$, *iha*, *est*Ia (STa), *paa*, *ter*E, and *ure*D. One strain, serotype O49:H21, carried the *eae* and virulence gene profile 12, which included two *nle* variants: *stx*$_{2e}$, *eae*, *nle*F, *nle*H1-2, *kat*P, *iha*, ecs1763, *lpf*A-O113, *ast*A, *est*Ia (STa), *ecf*1, *ecf*2, *ecf*3, *ecf*4, *irp*2, *fyu*A, *eha*A, *paa*, *ter*E, and *ure*D.

Although one out of the 155 strains carried the *eae* gene, which encodes attachment protein intimin, other attachment protein genes were present in the swine STEC strains. For example, the *iha* gene, which encodes the iron regulated gene A homologue adhesin (40), was detected in 99.4% (154/155) of the STEC strains. The *lpfA* –O113 gene which encodes long polar fimibriae (41) was detected in 85.8% (133/155) of the strains. The *fedA* gene, which encodes fimbrial adhesin F18 (42), was present in 0.6% (1/155) of the strains. The *orfA* and *orfB* genes, which encode adhesins involved in diffuse adherence (AIDA) (43, 44) were present in

1.3% (2/155) of the strains. Moreover, the *paa* gene, which encodes porcine attaching and effacing associated adhesion (45), was detected in 99.4% (154/155) of the strains.

A number of genes that encode toxins and hemolysins were present in the panel of swine STEC strains. For instance, 151 of 155 the swine STEC strains carried the stx_{2e} gene, and the other 4 of the 155 strains carried the stx_1 gene. The *ast*A gene, which encodes enteroaggregative *E. coli* heat stable toxin (46), was detected in 3.2% (5/155) of the strains. The *estIa* (*STa*) gene, which encodes heat stable toxin (47), was detected in 94.8% (147/155) of the strains. Moreover, the *hlyA* gene, which encodes the alpha hemolysin (48), was present in 0.6% (1/155) of the strains. Interestingly, the ecs1763 gene, which was suggested to be associated with enterohemorrhagic *E. coli* (EHEC) (49), was present in 85.2% (132/155) of the strains. The following virulence genes were not detected in any of the 155 swine STEC strains: *eae* subtypes alpha, beta, gamma, epsilon and theta, *nle*A, *nle*G5, *ent*/*esp*L2, *nle* B, *nle* E, *efa*1/*lif*A, Z2096, Z2098, Z2099, espM1, espM2, *nle*G6-2, *esp*K, *esp*N, *esp*X7, *esp*O1-1, *esp*V, ecs1822, *sfp, bfp, lpf*A-O26, *lpf*A-O157, *cdt* subtypes I and III, *elt*(LT), *fas*A, *fim*F, *cnf*2, *ehx*A, *tox*B, *stc*E, *eib*G, *epe*A, *esp*P, *saa*, *sub*AB, and *sab*.

PFGE

Within the O59:H21 STEC strains, three major clusters (clusters A-C) were defined at a cut-off value of 80% similarity (Figure 3.1). These three major clusters were related at a 71.1% similarity. The strains isolated from pigs within the same cohort were clustered together, for example, cluster A contained strains from pigs in cohort 3, and cluster B contained strains from pigs in cohort 1. The only one exception was that strain number 297 from pig number 119 in

cohort 3 clustered with strains from cohort 2 (in cluster C). Strains from pigs in cohort 3 were related to the strains from pigs in cohort 1 at a 75.1% similarity. Within each cluster, indistinguishable PFGE patterns were observed among STEC strains recovered from samples in the same pig over time during the finishing period. For example, strains 170 and 228 with indistinguishable PFGE patterns were recovered from pig number 145 at the second and third farm visits in cohort 3. The two O59:H19 strains, which carried virulence gene profile 3, were not clustered with the O59:H21 strains (24.8% similarity, data not shown).

Within the O untypeable:H19 strains, 11 out of the 13 strains were clustered together at a 83.6% similarity. One of the O untypeable:H19 strain contained distinguishable PFGE patterns from the other 11 strains (27.4% similarity). Moreover, strain 281 which carried a different virulence gene profile was not clustered with the other 12 strains (Figure 3.2). Within the five strains belonging to serogroup O98, the four O98:H12 strains had indistinguishable PFGE patterns and were clustered together at a similarity over 90%. The O98:H19 strain (n=1) was not clustered with the four O98:H12 strains (Figure 3.3).

DISCUSSION

The objective of this study was to use molecular methods to characterize swine STEC strains to assess their potential pathogenicity and determine their genetic relatedness. This is the first study to utilize a high throughput real-time PCR platform to examine the presence of a large panel of virulence gene targets in swine STEC strains. Given the fact that these swine STEC strains were recovered from samples of 97 healthy finishing pigs from three cohorts within 18 months, they are diverse with 16 different combinations of virulence gene profiles and serotypes. The panel of virulence gene targets in this study included 69 targets, and our results were in agreement with another study suggesting that increasing numbers of virulence genes in the panel will increase the resolution of the virulence gene profiling (50). Various virulence gene profiles in swine STEC strains have also been reported elsewhere (34-38, 51-53). However, it was challenging to compare results of this study with these previous reports because every study employed a different panel of virulence genes. Together, our results and the previous studies in swine STEC strains have indicated that swine STEC consist of strains carrying diverse virulence gene profiles.

Because the swine STEC strains used in this study belonged to non-O157 O serogroups, and only one (O49:H21) of the 155 strains carried the *eae* gene, we selected many novel virulence gene targets reported in non-O157 and LEE-negative STEC strains. Some attachment protein genes which have been detected in human pathogenic STEC strains were present in the swine STEC strains. For example, the *iha* gene and the *lpf*A –O113 gene were present in over 80% of the swine STEC strains. These two genes have been detected in LEE-negative STEC strains which were associated with human clinical cases (54-56). Unlike the intimin-encoding

eae gene, other attachment protein genes and their roles in the pathogenesis of STEC in humans have not been extensively studied. In addition to their potential ability to allow STEC to attach to human cells, the high prevalence of these genes in swine STEC strains may also suggest that they play a role in the colonization of STEC in swine population.

Interestingly, among all the swine STEC strains examined in this study, only one O untypeable:H4 strain carried the *fedA* gene, which encodes fimbrial adhesin F18 and is associated with swine edema disease (42). This finding may partially explain that all the pigs included in the longitudinal study were clinically healthy. Moreover, the presence of the many targets found in our panel of virulence genes has not been examined in swine STEC strains elsewhere. For example, the ecs1763 gene, which was only found in the EHEC strains analyzed in the previous study (49), was prevalent in a high proportion (85.2%) of the swine strains. However, the function associated with this ecs1763 gene has not yet been characterized, and the association between the presence of this gene and the clinical outcome in human cases requires more research. In addition, the combination of *espK* with either *espV*, *ureD*, or Z2098 has been suggested to be highly prevalent in EHEC strains, and can be utilized for identifying typical EHEC strains which are both *stx*- and *eae*-positive (57). Although *ureD* was present in 99.4% of our strains, the *espK*, *espV*, and Z2098 genes were absent in the panel of strains used in this study.

One of the challenges in this study was that some of the primers in the real-time PCR system used in this study may not detect genetic variation in some of the genes. For example, the O49:H21 STEC which was positive for the *eae*, but was negative for the five *eae* variants examined by this real-time PCR system. Therefore, misclassification of a strain as negative for certain genes might occur when some strains carried genes with variants that the primers

included in this real-time PCR system were not able to target. Therefore, further validation of the specificity or sensitivity of this PCR system is warranted. Another challenge in interpreting virulence gene profiles is that there has not been a "gold standard" virulence gene profile identified that accurately predicts virulence and pathogenicity of STEC strains in humans (58). This challenge may result from multiple factors. For example, functions of many of the associated virulence factors need further characterization, and much is unknown about the interactions of STEC and human hosts. Notably, much of the current understanding about STEC pathogenesis is based on STEC serotype O157:H7 and the LEE-positive STEC strains. To be able to better interpret these virulence gene profiles and to elucidate their association with pathogenicity, it is critical to understand the whole picture of STEC pathogenesis in humans and colonization in both humans and their animal hosts. One of the many future directions is to use cell culture assays to understand the interaction of these swine STEC strains with human epithelial cell lines. For instance, it has been suggested that swine O157:H7 STEC strains affect the integrity of human epithelial cells (59). Considering the uncertainties of some of these putative virulence factors in causing human illness, it is difficult to determine the health risk of many of these swine STEC strains. However, one may notice that some swine STEC strains had only partial serotypes and most of those that were identified belonged to less common serotypes.

This is the first study to use PFGE to analyze STEC strains recovered from repeated samples from pigs in a longitudinal study. Most of the previous studies used PFGE to determine the genetic relatedness of STEC strains from swine to other species (53, 60-63). These results were inconsistent, and most studies focused on STEC O157:H7 (53, 60, 62, 63). From the PFGE results, the O59:H21 STEC strains, which were predominant in the current swine STEC collection, were closely related to other strains from pigs within the same cohort at greater than

80% similarity. This may suggest that the same strain disseminated within each cohort, supporting that there might be a point-source outbreak within each cohort which was raised on three different barns at two finishing sites (M. Tseng, P. M. Fratamico, L. Bagi, D. *Manzinger*, and J. A. Funk, in press). For example, the pigs were exposed to the same point source of infections in the finishing site environment. This cohort-specific PFGE pattern was also observed in a study, which was conducted in cattle (64). More research is needed to investigate potential risk factors and the common source of infection associated with STEC strains shed by finishing swine.

It was observed that swine STEC strains of the same serotype can carry different virulence gene profiles. For example, there were two virulence gene profiles (1 and 2) within O59:H21 strains. However, the strains with different virulence gene profiles were clonally related by PFGE analysis. This was not unexpected because the two virulence gene profiles (1 and 2) were different by one gene *est*Ia (STa), which is carried on a plasmid. Moreover, PFGE can determine the genetic relatedness of the swine STEC strains, but cannot provide information regarding the genetic components of the patterns (65). Future studies using sequence-based molecular methods, for example, whole genome sequencing, can provide us more insights into the genetic diversity of swine STEC. The results of this study help fill in the current knowledge gap regarding swine STEC. Our results demonstrated the diverse virulence genes carried by swine STEC and closely-related strains shed by pigs in the same cohort. However, whether these swine STEC strains are potentially pathogenic to humans and the role swine play in the transmission of STEC to humans require more research endeavors.

ACKNOWLEDGEMENTS

The work was supported by the National Pork Board (grant number 12-069) and U.S. Department of Agriculture, National Institute of Food and Agriculture, Agriculture and Food Research Initiative (award number 2013-67005-21189). The PCR microarray development was partially financed by the French "joint ministerial program of R&D against CBRNE risks".

Sincerely thanks to Dr. Chitritia DebRoy, and the *E. coli* Reference Center at Pennsylvania State University for assisting with characterization of the swine STEC strains, Niesa Kettler, Terrence MacManus, and the Bacteriology Branch in Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University for assisting with the PFGE process.

APPENDIX

Table 3.1. Virulence gene targets, their encoded proteins and locations

virulence gene targets	proteins encoded	location	GenBank accession number
stx - all variants	Shiga toxin	chromosome	NC002695
stx_{2e}	Shiga toxin 2e variant	chromosome	AB252836
eae	Intimin	chromosome, LEE (locus of enterocyte effacement)	Z11541
eae-alpha			
eae-beta			
eae-gamma			
eae-epsilon			
eae-theta			
iha	Iha, iron regulated gene A homolog, adhesion	chromosome, OI-43, OI-48	AF126104
*ter*E	tellurite resistant gene	chromosome, OI-43, OI-48	CP003301
*ure*D	urase transporter	chromosome, OI-43, OI-48	AE005174
*esp*V	AvrA family effectors	chromosome, OI-44	AE005174

Table 3.1. (cont'd)

virulence gene targets	proteins encoded	location	GenBank accession number
*esp*K	non-LEE-encoded type III secretion system effectors	chromosome, OI-50	AE005174
*esp*N	non-LEE-encoded type III secretion system effectors	chromosome, OI-50	AE005174
*esp*X7	non-LEE-encoded type III secretion system effectors	chromosome, OI-50	AE005174
*esp*O1-1	non-LEE-encoded type III secretion system effectors	chromosome, OI -50	AE005174
*nle*G5	non-LEE-encoded type III secretion system effectors	chromosome, OI -57	AE005174
*nle*G6-2	non-LEE-encoded type III secretion system effectors	chromosome, OI -57	AE005174
Z2096	putative gene marker for EHEC	chromosome, OI-57	AE005174
Z2098	putative gene marker for EHEC	chromosome, OI-57	AE005174

Table 3.1. (cont'd)

virulence gene targets	proteins encoded	location	GenBank accession number
Z2099	putative gene marker for EHEC	chromosome, OI-57	AE005174
*nle*A	serine protease	chromosome, OI-71	AE005174
*nle*F	non-LEE-encoded type III secretion system effectors	chromosome, OI-71	AE005174
*nle*H1-2	NleH	chromosome, OI-71	AE005174
*esp*M1	non-LEE-encoded type III secretion system effectors	chromosome, OI-71	AE005174
*esp*M2	IpgM	chromosome, OI-108	AE005174
*nle*B	non-LEE-encoded type III secretion system effectors	chromosome, OI-122	AE005174
*nle*E	non-LEE-encoded type III secretion system effectors	chromosome, OI-122	AE005174
efa1(*lif*A)	Efa1 EHEC factor for adherence	chromosome, OI-122	AE005174

Table 3.1. (cont'd)

virulence gene targets	proteins encoded	location	GenBank accession number
*ent/esp*L2	Enterotoxin	chromosome, OI-122	AE005174
*pag*C	phoP-activated gene C product (PagC)	chromosome, OI-122	AE005174
*lpf*A-O157	long polar fimbriae	chromosome, OI-141, OI-154	AE005174
*lpf*A-O113	long polar fimbriae	chromosome, OI-154	AY057066
*lpf*A-O26	long polar fimbriae	chromosome	AB161111
ecs1822	hypothetical protein, putative gene marker for EHEC	chromosome	BA000007
ecs1763	hypothetical protein, putative gene marker for EHEC	chromosome	BA000007
*irp*2	iron responsible protein 2	chromosome, Yersinia-like high pathogenecity island	CP000468
*fyu*A	pectin receptor	chromosome, Yersinia-like high pathogenecity island	AFST01000010

Table 3.1. (cont'd)

virulence gene targets	proteins encoded	location	GenBank accession number
*eha*A	autotransporter, type V secretion system	chromosome	AE005174
*hly*A	alpha hemolysin	chromosome	AE014075
paa	porcine attaching and effacing associated adhesion	chromosome or plasmid	U82533
*cdt*I	cytolethal distending toxin	chromosome or plasmid	AY365042
*cdt*III	cytolethal distending toxin	chromosome or plasmid	AY365042
*ast*A	enteroaggregative *E. coli* heat stable enterotoxin	chromosome or plasmid	L11241
*est*Ia (STa)	heat stable toxin	plasmid	M58746
elt (LT)	heat liable toxin	plasmid	KF733765
sfp	sorbitol-fermenting protein fimbriae	plasmid	AF401292
bfp	bundle-forming pilus	plasmid	AB024946

Table 3.1. (cont'd)

virulence gene targets	proteins encoded	location	GenBank accession number
*fas*A (F6, P987)	F6 fimbrial adhesion	plasmid	U50547
*fed*A (F18, F107)	F18 fimbrial adhesion	plasmid	M61713
$fimF_{41a}$ (F41)	F41 fimbrial adhesion	plasmid	X14354
*cnf*2	cytotoxic necrotizing factor	Vir plasmid	U01097
*orf*A	adhesin invovled in diffuse adherence (AIDA)	plasmid	X65022
*orf*B	adhesin invovled in diffuse adherence (AIDA)	plasmid	X65022
*ecf*1	enzyme that enhance bacterial membrane structure	plasmid O157	AF043470
*ecf*2	enzyme that enhance bacterial membrane structure	plasmid O157	AF043470
*ecf*3	enzyme that enhance bacterial membrane structure	plasmid O157	AF043470

Table 3.1. (cont'd)

virulence gene targets	proteins encoded	location	GenBank accession number
*ecf*4	enzyme that enhance bacterial membrane structure	plasmid O157	AF043470
*kat*P	catalase/peroxidase	plasmid O157	X89017
*ehx*A	EHEC hemolysin	plasmid O157	AF074613
*tox*B	potential adhesin for adherence,	plasmid O157	AB011549
*etp*D	EHEC type II secretion system, transporting protein across the outer membrane	plasmid O157	CP001163
*stc*E	metalloprotease, mucinase	plasmid O157	AF074613
espP	serine protease autotransporter	plasmid O157	CP001163
*eib*G	immunoglobulin binding protein	plasmid O113	AB255744
epeA	EpeA, serine protease	plasmid O113	AY258503

Table 3.1. (cont'd)

virulence gene targets	proteins encoded	location	GenBank accession number
saa	Saa, STEC agglutinating adhesion	plasmid O113	AF399919
*sub*AB	subtilase cytotoxin	plasmid O113	AF399919
sab	autotransporter	plasmid O113	AF399919

Table 3.2. Distribution of swine STEC strains by serotype and virulence gene profiles

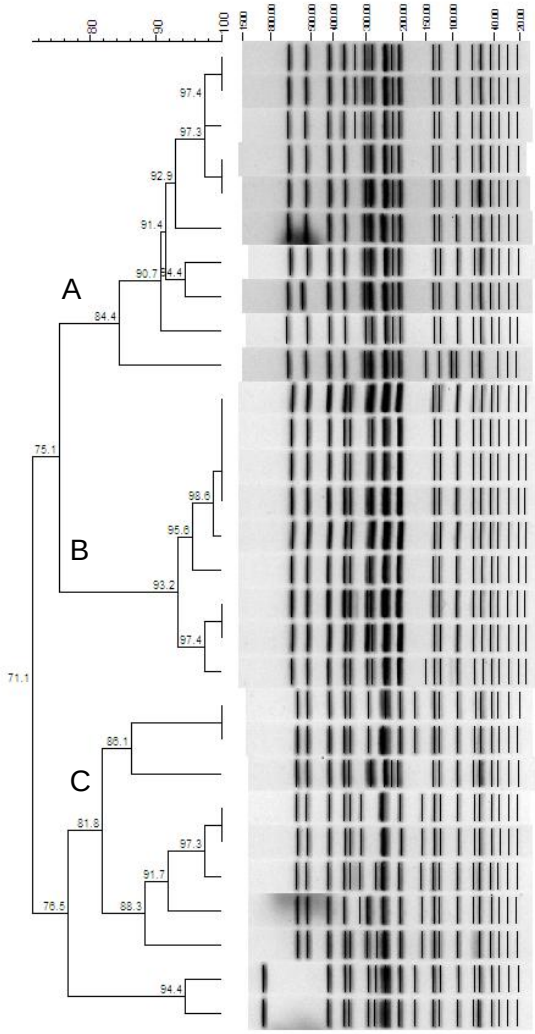
Serotype	numbers of swine STEC strains	numbers of strains analyzed by PFGE	virulence gene profile	virulence gene profile code
O59:H21	120	27	*stx*$_{2e}$, *iha*, ecs1763, *lpf*A-O113, *est*Ia (STa), *eha*A, *paa*, *ter*E, *ure*D	1
	2	2	*stx*$_{2e}$, *iha*,ecs1763, *lpf*A-O113, *eha*A, *paa*, *ter*E, *ure*D	2
	3	3	*stx*$_{2e}$, *etp*D, *iha*, ecs1763, *lpf*A-O113, *est*Ia (STa), *ecf*1, *ecf*2, *ecf*3, *ecf*4, *eha*A, *paa*, *ter*E, *ure*D	11
O untypeable:H19	13	12	*stx*$_{2e}$, *iha*, *est*Ia (STa), *paa*, *ter*E, *ure*D	3
	1	1	*stx*$_{2e}$, *iha*, *ast*A, *est*Ia (STa), *ter*E, *ure*D	4
O59:H19	2	2	*stx*$_{2e}$, *iha*, *est*Ia (STa), *paa*, *ter*E, *ure*D	3

Table 3.2. (cont'd)

Serotype	numbers of swine STEC strains	numbers of strains analyzed by PFGE	virulence gene profile	virulence gene profile code
O98:H12	2	2	*stx*$_1$, *pag*C, *kat*P, *iha*, *ast*A, *ecf*1, *ecf*2, *ecf*3,*ecf*4, *paa*, *ter*E, *ure*D	9
	2	2	*stx*$_1$, *pag*C, *kat*P, *iha*, *ecf*1, *ecf*2, *ecf*3,*ecf*4, *paa*, *ter*E, *ure*D	10
O untypeable:H21	2	0	*stx*$_{2e}$, *iha*, ecs1763, *lpf*A-O113, *est*Ia (STa), *eha*A, *paa*, *ter*E, *ure*D	1
O20:H21	1	0	*stx*$_{2e}$, *iha*, ecs1763, *lpf*A-O113, *est*Ia (STa), *eha*A, *paa*, *ter*E, *ure*D	1
O49:H21	1	0	*stx*$_{2e}$, *eae*, *nle*F, *nle*H1-2, *kat*P, *iha*,ecs1763, *lpf*A-O113, *ast*A, *est*Ia (STa), *ecf*1, *ecf*2, *ecf*3, *ecf*4, *irp*2, *fyu*A, *eha*A, *paa*, *ter*E, *ure*D	12

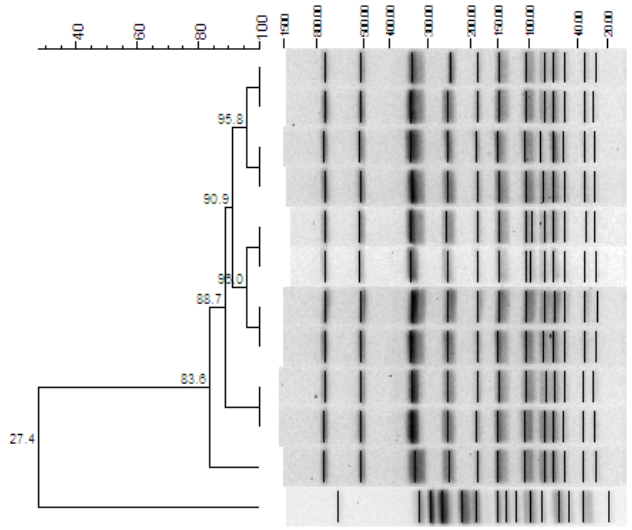
Table 3.2. (cont'd)

Serotype	numbers of swine STEC strains	numbers of strains analyzed by PFGE	virulence gene profile	virulence gene profile code
O89:H19	1	0	*stx*$_{2e}$, *iha*, *lpf*A-O113, *est*Ia (STa), *eha*A, *paa*, *ter*E, *ure*D	7
O98:H19	1	1	*stx*$_{2e}$, *pag*C, *kat*P , *iha*, *ecf*1, *ecf*2, *ecf*3, *ecf*4, *paa*, *ter*E, *ure*D	5
O115:H19	1	0	*stx*$_{2e}$, *iha*, ecs1763, *lpf*A-O113, *ast*A, *est*Ia (STa),*orf*A, *orf*B, *eha*A, *paa*, *ter*E, *ure*D	6
O119:H21	1	0	*stx*$_{2e}$, *iha*, ecs1763, *lpf*A-O113, *est*Ia (STa), *eha*A, *paa*, *ter*E, *ure*D	1
O167:H21	1	0	*stx*$_{2e}$, *iha*, ecs1763, *lpf*A-O113, *est*Ia (STa), *eha*A, *paa*, *ter*E, *ure*D	1
O untypeable:H4	1	0	*stx*$_{2e}$, *fed*A(F18), *hly*A, *orf*A, *orf*B, *paa*, *ter*E	8
Total	155	56		



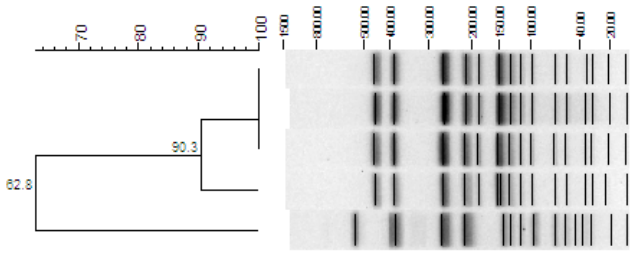
Key	Source (cohort, visit, pig number)	Serotype	Virulence gene profile
182	C3, V3, #105	O59:H21	2
184	C3, V3, #105	O59:H21	1
201	C3, V3, #119	O59:H21	2
170	C3, V2, #145	O59:H21	1
185	C3, V3, #106	O59:H21	1
228	C3, V3, #145	O59:H21	1
192	C3, V3, #109	O59:H21	1
256	C3, V4, #118	O59:H21	1
300	C3, V6, #118	O59:H21	1
277	C3, V4, #137	O59:H21	1
120	C1, V6, #49	O59:H21	1
2	C1, V3, #31	O59:H21	1
3	C1, V4, #1	O59:H21	1
46	C1, V4, #27	O59:H21	1
25	C1, V4, #19	O59:H21	1
84	C1, V5, #25	O59:H21	1
118	C1, V6, #19	O59:H21	1
36	C1, V4, #25	O59:H21	1
71	C1, V5, #1	O59:H21	1
113	C2, V2, #100	O59:H21	1
123	C2, V3, #76	O59:H21	1
297	C3, V5, #119	O59:H21	1
104	C2, V7, #67	O59:H21	1
145	C2, V4, #64	O59:H21	1
11	C2, V5, #64	O59:H21	1
156	C2, V4, #82	O59:H21	1
151	C2, V4, #72	O59:H21	1
132	C2, V3, #100	O59:H21	1
183	C2, V4, #100	O59:H21	1

Figure 3.1. PFGE analysis of swine O59:H21 STEC strains. Key represents strain numbers. Source represents the strain from a pig in which cohort, which time of the eight farm visits, and the individual pig number.



Key	Source (cohort, visit, pig number)	Serotype	Virulence gene profile
280	C3, V4, #139	O untypeable:H19	3
282	C3, V4, #146	O untypeable:H19	3
248	C3, V4, #107	O untypeable:H19	3
267	C3, V4, #124	O untypeable:H19	3
306	C3, V6, #141	O untypeable:H19	3
314	C3, V8, #119	O untypeable:H19	3
253	C3, V4, #112	O untypeable:H19	3
254	C3, V4, #114	O untypeable:H19	3
229	C3, V3, #145	O untypeable:H19	3
240	C3, V4, #104	O untypeable:H19	3
252	C3, V4, #109	O untypeable:H19	3
281	C3, V4, #145	O untypeable:H19	4

Figure 3.2. PFGE analysis of swine O untypeable:H19 STEC strains. Key represents strain numbers. Source represents the strain from a pig in which cohort, which time of the eight farm visits, and the individual pig number.



Key	Source (cohort, visit, pig number)	Serotype	Virulence gene profile
301	C3, V6, #118	O98:H12	10
302	C3, V6, #123	O98:H12	10
308	C3, V7, #134	O98:H12	9
108	C2, V7, #75	O98:H12	9
206	C3, V3, #122	O98:H19	5

Figure 3.3. PFGE analysis of swine O98 STEC strains. Key represents strain numbers. Source represents the strain from a pig in which cohort, which time of the eight farm visits, and the individual pig number

REFERENCES

REFERENCES

1. Scallan, E., et al., *Foodborne Illness Acquired in the United States—Major Pathogens.* Emerging Infectious Diseases, 2011. **17**(1): p. 7-15.

2. Hoffmann, S., M.B. Batz, and J.G. Morris, *Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens.* Journal of Food Protection, 2012. **75**(7): p. 1292-1302.

3. Unkmeir, A. and H. Schmidt, *Structural analysis of phage-borne stx genes and their flanking sequences in shiga toxin-producing Escherichia coli and Shigella dysenteriae type 1 strains.* Infection and Immunity, 2000. **68**(9): p. 4856-64.

4. O'Brien, A.D., et al., *Environmental and human isolates of Vibrio cholerae and Vibrio parahaemolyticus produce a Shigella dysenteriae 1 (Shiga)-like cytotoxin.* Lancet, 1984. **1**(8368): p. 77-8.

5. Nataro, J.P. and J.B. Kaper, *Diarrheagenic Escherichia coli.* Clin Microbiol Rev, 1998. **11**(1): p. 142-201.

6. Rangel, J.M., et al., *Epidemiology of Escherichia coli O157:H7 outbreaks, United States, 1982-2002.* Emerg Infect Dis, 2005. **11**(4): p. 603-9.

7. Gyles, C.L., *Shiga toxin-producing Escherichia coli: an overview.* J Anim Sci, 2007. **85**(13 Suppl): p. E45-62.

8. Paton, A.W., et al., *Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing Escherichia coli.* J Clin Microbiol, 1996. **34**(7): p. 1622-7.

9. CDC, *Escherichia coli O157:H7 outbreak linked to commercially distributed dry-cured salami- Washington and California, 1994.* Morbidity and Mortality Weekly Report, 1995. **44**: p. 157-160.

10. CDC, *Community outbreak of hemolytic uremic syndrome attributable to Escherichia coli O111:NM-South Australia, 1995.* Morbidity and Mortality Weekly Report, 1995. **44**: p. 550-558.

11. Williams, R.C., et al., *Illness outbreak associated with Escherichia coli O157:H7 in Genoa salami. E. coli O157:H7 Working Group.* Canadian Medical Association Journal, 2000. **162**(10): p. 1409-13.

12. MacDonald, D.M., et al., *Escherichia coli O157:H7 outbreak linked to salami, British Columbia, Canada, 1999.* Epidemiol Infect, 2004. **132**(2): p. 283-9.

13. Conedera, G., et al., *A family outbreak of Escherichia coli O157 haemorrhagic colitis caused by pork meat salami.* Epidemiol Infect, 2007. **135**(2): p. 311-4.

14. Trotz-Williams, L.A., et al., *Pork Implicated in a Shiga Toxin-producing Escherichia coli O157:H7 Outbreak in Ontario, Canada.* Canadian Journal of Public Health. Revue Canadienne de Sante Publique, 2012. **103**(5): p. e322-6.

15. Fairbrother, J.M., Gyles CL, *Colibacillosis*, in *Diseases of Swine*, L.K. Jeffrey Zimmerman, Alejandro Ramirez, Kent Schwartz,Gregory Stevenson Editor. 2012, John Wiley and sons, Inc.: West Sussex, UK. p. 723-747.

16. Fratamico, P.M., et al., *Prevalence and characterization of Shiga toxin-producing Escherichia coli in swine feces recovered in the National Animal Health Monitoring System's Swine 2000 study.* Appl Environ Microbiol, 2004. **70**(12): p. 7173-8.

17. Oporto, B., et al., *Escherichia coli O157:H7 and non-O157 Shiga toxin-producing E. coli in healthy cattle, sheep and swine herds in Northern Spain.* Zoonoses Public Health, 2008. **55**(2): p. 73-81.

18. Borie, C., et al., *Prevalence and characterization of enterohaemorrhagic Escherichia coli isoated from healthy cattle and pigs slaughtered in Santiago, Chile.* Archivos De Medicina Veterinaria, 1997. **29**(2): p. 205-212.

19. Karmali, M.A., et al., *Escherichia coli cytotoxin, haemolytic-uraemic syndrome, and haemorrhagic colitis.* Lancet, 1983. **2**(8362): p. 1299-1300.

20. Kaper, J.B., J.P. Nataro, and H.L. Mobley, *Pathogenic Escherichia coli.* Nat Rev Microbiol, 2004. **2**(2): p. 123-40.

21. Croxen, M.A. and B.B. Finlay, *Molecular mechanisms of Escherichia coli pathogenicity.* Nat Rev Microbiol, 2010. **8**(1): p. 26-38.

22. Melton-Celsa, A., et al., *Pathogenesis of Shiga-toxin producing escherichia coli.* Curr Top Microbiol Immunol, 2012. **357**: p. 67-103.

23. Petruzziello-Pellegrini, T.N., M. Moslemi-Naeini, and P.A. Marsden, *New insights into Shiga toxin-mediated endothelial dysfunction in hemolytic uremic syndrome.* Virulence, 2013. **4**(6): p. 556-63.

24. Croxen, M.A., et al., *Recent Advances in Understanding Enteric Pathogenic Escherichia coli.* Clinical Microbiology Reviews, 2013. **26**(4): p. 822-80.

25. Endo, Y., et al., *Site of action of a Vero toxin (VT2) from Escherichia coli O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins.* European Journal of Biochemistry, 1988. **171**(1-2): p. 45-50.

26. Johannes, L. and W. Romer, *Shiga toxins--from cell biology to biomedical applications.* Nat Rev Microbiol, 2010. **8**(2): p. 105-16.

27. Bugarel, M., et al., *Virulence gene profiling of enterohemorrhagic (EHEC) and enteropathogenic (EPEC) Escherichia coli strains: a basis for molecular risk assessment of typical and atypical EPEC strains.* BMC Microbiol, 2011. **11**: p. 142.

28. Low, A.S., et al., *Analysis of fimbrial gene clusters and their expression in enterohaemorrhagic Escherichia coli O157:H7.* Environ Microbiol, 2006. **8**(6): p. 1033-47.

29. Frankel, G. and A.D. Phillips, *Attaching effacing Escherichia coli and paradigms of Tir-triggered actin polymerization: getting off the pedestal.* Cell Microbiol, 2008. **10**(3): p. 549-56.

30. Paton, A.W., et al., *Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic Escherichia coli strains that are virulent for humans.* Infect Immun, 2001. **69**(11): p. 6999-7009.

31. Brunder, W., H. Schmidt, and H. Karch, *KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic Escherichia coli O157:H7.* Microbiology, 1996. **142 (Pt 11)**: p. 3305-15.

32. Coombes, B.K., et al., *Molecular analysis as an aid to assess the public health risk of non-O157 Shiga toxin-producing Escherichia coli strains.* Appl Environ Microbiol, 2008. **74**(7): p. 2153-60.

33. Slanec, T., et al., *Molecular analysis of virulence profiles and Shiga toxin genes in food-borne Shiga toxin-producing Escherichia coli.* Appl Environ Microbiol, 2009. **75**(19): p. 6187-97.

34. Fratamico, P.M., et al., *Characterization of Shiga toxin-producing Escherichia coli strains isolated from swine feces.* Foodborne Pathogen and Disease, 2008. **5**(6): p. 827-38.

35. Meng, Q., et al., *Characterization of Shiga toxin-producing Escherichia coli isolated from healthy pigs in China.* BMC Microbiol, 2014. **14**: p. 5.

36. Chapman, T.A., et al., *Comparison of virulence gene profiles of Escherichia coli strains isolated from healthy and diarrheic swine.* Appl Environ Microbiol, 2006. **72**(7): p. 4782-95.

37. Zweifel, C., et al., *Virulence profiles of Shiga toxin 2e-producing Escherichia coli isolated from healthy pig at slaughter.* Vet Microbiol, 2006. **117**(2-4): p. 328-32.

38. Sonntag, A.K., et al., *Shiga toxin 2e-producing Escherichia coli isolates from humans and pigs differ in their virulence profiles and interactions with intestinal epithelial cells.* Appl Environ Microbiol, 2005. **71**(12): p. 8855-63.

39. Ribot, E.M., et al., *Standardization of pulsed-field gel electrophoresis protocols for the subtyping of Escherichia coli O157:H7, Salmonella, and Shigella for PulseNet.* Foodborne Pathog Dis, 2006. **3**(1): p. 59-67.

40. Tarr, P.I., et al., *Iha: a novel Escherichia coli O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure.* Infect Immun, 2000. **68**(3): p. 1400-7.

41. Doughty, S., et al., *Identification of a novel fimbrial gene cluster related to long polar fimbriae in locus of enterocyte effacement-negative strains of enterohemorrhagic Escherichia coli.* Infect Immun, 2002. **70**(12): p. 6761-9.

42. Frydendahl, K., *Prevalence of serogroups and virulence genes in Escherichia coli associated with postweaning diarrhoea and edema disease in pigs and a comparison of diagnostic approaches.* Vet Microbiol, 2002. **85**(2): p. 169-82.

43. Benz, I. and M.A. Schmidt, *Isolation and serologic characterization of AIDA-I, the adhesin mediating the diffuse adherence phenotype of the diarrhea-associated Escherichia coli strain 2787 (O126:H27).* Infection and Immunity, 1992. **60**(1): p. 13-8.

44. Benz, I. and M.A. Schmidt, *Cloning and expression of an adhesin (AIDA-I) involved in diffuse adherence of enteropathogenic Escherichia coli.* Infection and Immunity, 1989. **57**(5): p. 1506-11.

45. An, H., et al., *Distribution of a novel locus called Paa (porcine attaching and effacing associated) among enteric Escherichia coli.* Advances in Experimental Medicine and Biology, 1999. **473**: p. 179-84.

46. Osek, J., *Detection of the enteroaggregative Escherichia coli heat-stable enterotoxin 1 (EAST1) gene and its relationship with fimbrial and enterotoxin markers in E. coli isolates from pigs with diarrhoea.* Veterinary Microbiology, 2003. **91**(1): p. 65-72.

47. Blanco, M., et al., *Genes coding for enterotoxins and verotoxins in porcine Escherichia coli strains belonging to different O:K:H serotypes: relationship with toxic phenotypes.* Journal of Clinical Microbiology, 1997. **35**(11): p. 2958-63.

48. Welch, R.A., et al., *The synthesis and function of the Escherichia coli hemolysin and related RTX exotoxins.* FEMS Microbiology Immunology, 1992. **5**(1-3): p. 29-36.

49. Abu-Ali, G.S., et al., *Genomic diversity of pathogenic Escherichia coli of the EHEC 2 clonal complex.* BMC Genomics, 2009. **10**: p. 296.

50. Brandt, S.M., et al., *Molecular risk assessment and epidemiological typing of Shiga toxin-producing Escherichia coli by using a novel PCR binary typing system.* Appl Environ Microbiol, 2011. **77**(7): p. 2458-70.

51. Kaufmann, M., et al., *Escherichia coli O157 and non-O157 Shiga toxin-producing Escherichia coli in fecal samples of finished pigs at slaughter in Switzerland.* J Food Prot, 2006. **69**(2): p. 260-6.

52. Ateba, C.N. and M. Mbewe, *Detection of Escherichia coli O157:H7 virulence genes in isolates from beef, pork, water, human and animal species in the northwest province, South Africa: public health implications.* Res Microbiol, 2011. **162**(3): p. 240-248.

53. Lenahan, M., et al., *Molecular characterization of Irish E. coli O157:H7 isolates of human, bovine, ovine and porcine origin.* Journal of Applied Microbiology, 2009. **107**(4): p. 1340-1349.

54. Newton, H.J., et al., *Shiga toxin-producing Escherichia coli strains negative for locus of enterocyte effacement.* Emerging Infectious Diseases, 2009. **15**(3): p. 372-80.

55. Hauser, E., et al., *Phylogenetic and molecular analysis of food-borne shiga toxin-producing Escherichia coli.* Applied and Environmental Microbiology, 2013. **79**(8): p. 2731-40.

56. Galli, L., et al., *Virulence profile comparison between LEE-negative Shiga toxin-producing Escherichia coli (STEC) strains isolated from cattle and humans.* Veterinary Microbiology, 2010. **143**(2-4): p. 307-13.

57. Delannoy, S., L. Beutin, and P. Fach, *Discrimination of enterohemorrhagic Escherichia coli (EHEC) from non-EHEC strains based on detection of various combinations of type III effector genes.* Journal of Clinical Microbiology, 2013. **51**(10): p. 3257-62.

58. Anjum, M.F., et al., *Use of virulence determinants and seropathotypes to distinguish high- and low-risk Escherichia coli O157 and non-O157 isolates from Europe.* Epidemiology and Infection, 2013: p. 1-10.

59. Kennedy, C.A., et al., *Shiga toxin-producing Escherichia coli isolated from human and pig origin induce different gene expression profiles in human Caco-2 epithelial cells.* Livestock Science, 2010. **133**(1/3): p. 189-191.

60. Johnsen, G., et al., *Escherichia coli O157:H7 in faeces from cattle, sheep and pigs in the southwest part of Norway during 1998 and 1999.* Int J Food Microbiol, 2001. **65**(3): p. 193-200.

61. Franke, S., et al., *Clonal relatedness of Shiga-like toxin-producing Escherichia coli O101 strains of human and porcine origin.* J Clin Microbiol, 1995. **33**(12): p. 3174-8.

62. Osek, J. and P. Gallien, *Molecular analysis of Escherichia coli O157 strains isolated from cattle and pigs by the use of PCR and pulsed-field gel electrophoresis methods.* Veterinarni Medicina, 2002. **47**(6): p. 149-158.

63. Rios, M., et al., *Clonal diversity of Chilean isolates of enterohemorrhagic Escherichia coli from patients with hemolytic-uremic syndrome, asymptomatic subjects, animal reservoirs, and food products.* J Clin Microbiol, 1999. **37**(3): p. 778-81.

64. Joris, M.A., et al., *Longitudinal follow-up of the persistence and dissemination of EHEC on cattle farms in Belgium.* Foodborne Pathog Dis, 2013. **10**(4): p. 295-301.

65. Singer, R.S., W.M. Sischo, and T.E. Carpenter, *Exploration of biases that affect the interpretation of restriction fragment patterns produced by pulsed-field gel electrophoresis.* J Clin Microbiol, 2004. **42**(12): p. 5502-11.

CHAPTER 4

Shiga toxin-producing *E. coli* (STEC) cases in Michigan, U.S., 2001-2012

ABSTRACT

Infection with Shiga toxin-producing *Escherichia coli* (STEC) by serotypes other than O157 (non-O157) have been increasingly reported as the cause of human illness in the U.S. This increase in reporting is likely primarily due to the improvements in diagnostic tests. To better understand the demographic characteristics of STEC cases and the odds of hospitalization associated with STEC infections, specifically non-O157 STEC, this study reviewed STEC cases reported to the Michigan Department of Community Health (MDCH) through the Michigan Disease Surveillance System (MDSS) from 2001 through 2012. An increasing trend of non-O157 STEC cases was observed in this 12-year period, and the incidence rates were similar for O157 and non-O157 STEC cases in 2012. No demographic characteristics were significantly different between O157 and non-O157 STEC cases. However, the odds of hospitalization were 2.36 times higher in O157 STEC cases than in non-O157 STEC cases when adjusted for age and gender. The information enhances our understanding in epidemiology of non-O157 STEC in Michigan, and future research is warranted to understand these pathogens in order to improve prevention and control efforts.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) represent a subset of *E. coli* strains that are capable of producing one or more Shiga toxins (Stx). STEC infections, mostly acquired from consuming contaminated food or water, are associated with outbreaks and sporadic cases of diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) (1, 2). Among the HC cases, 3-9 % of sporadic cases to 20 % of epidemic cases develop the life-threatening HUS (3-5), which is one of the leading causes of acute renal failure in young children worldwide (3, 6, 7). Therefore, STEC infections are a critical public health concern, and more than 170,000 illnesses are attributed to STEC in the U.S. annually (8).

While STEC strains belonging to serotype O157 are more common among outbreaks and severe clinical cases (9), infections with non-O157 STEC strains have been increasingly documented (9-12). Non-O157 STEC infections have been viewed as a public health concern. Since the year 2000, non-O157 STEC has become a nationally notifiable disease in the U.S. (13). Six non-O157 STEC serogroups, namely O26, O103, O111, O121, O45, O145, are the most prevalent in human infections and thus, are viewed as a major public health concern. Recently, these six O serogroups joined O157 in their classification as adulterants in raw, non-intact beef products in the U.S. (14).

The lower reported rates of non-O157 STEC cases in the past may in part be explained by the challenges of non-O157 STEC detection methods. Over time the Center for Disease Control and Prevention (CDC) has updated recommendations for clinical laboratory practices to better detect non-O157 STEC infections (15-17). According to the CDC's FoodNet, which is an active surveillance system for laboratory-confirmed STEC cases in 10 states in the U.S., increasing

detection of non-O157 STEC infections has been associated with the improvement of laboratory detection protocols (18). Similar observations were also reported in studies conducted in states not part of the FoodNet, for example, in Washington (19) and a surveillance study conducted in 20 clinical laboratories in Michigan (20).

The association of non-O157 STEC with milder clinical symptoms may also contribute to the lower reported rates of non-O157 STEC (21). Having milder clinical symptoms decreases the chance of seeking medical attention, and this leads to a smaller chance of having the specimen collected and tested. Therefore, these cases with milder clinical symptoms are less likely to be diagnosed and reported to the public health agencies (22). Compared with O157 STEC cases, non-O157 STEC infections are less likely to lead to severe clinical symptoms, specifically bloody diarrhea, HUS, and resulting in hospitalization (11, 21, 23). However, it was estimated that more than 60% of STEC infections are attributed to non-O157 STEC in the U.S. (8).

Besides clinical symptoms, similar demographic characteristics associated with non-O157 STEC cases have been described in a number of studies. In general, more female cases were observed in both O157 STEC and non O-157 STEC infections (13, 20, 21, 23, 24). The age distribution was similar between O157 STEC cases and non-O157 STEC cases in the CDC's FoodNet data (13). In contrast, one study suggested that non-O157 STEC infections were more commonly identified in children younger than 5 years old (24). Relative to O157 STEC infections, however, little is known about the epidemiology of non-O157 STEC infections in human clinical cases.

Current disease control and prevention plans can be improved by enhancing our understanding of host characteristics associated with STEC infections and disease trends over time. Because Michigan is not included in the CDC's FoodNet records, this study was conducted

to examine the epidemiology of STEC cases reported to the Michigan Department of Community Health (MDCH) over a 12-year period. Trends associated with STEC case were examined per year, as were the demographic and clinical data associated with each STEC case.

MATERIAL AND METHODS

Data source

The STEC case information was retrieved from the Michigan Disease Surveillance System (MDSS), managed by the MDCH (25). MDSS is a web-based surveillance system in which healthcare providers and clinical laboratories report communicable diseases to the public health system. Within 24 hours of STEC identification, the case must be reported to the local health department via MDSS. The MDCH is simultaneously notified via the MDSS when the case is reported to the local health department. The MDCH then reports the case to the CDC. All STEC serotypes are reportable, and an STEC isolate (if available) or non-culture positive broth and stool sample must be submitted to MDCH Bureau of Laboratories for isolation, confirmation and genotyping (26).

The MDSS has been designed according to U.S. national data standards. An electronic case investigation form includes the following information (if available): patient contact information, demographic, hospitalization status, clinical symptoms, laboratory data (e.g. serotype, virulence gene profiles, Shiga toxin information), collection date, length of illness, outbreak status, and notes containing additional clinical and/or follow-up information, for example, updated STEC isolate serotyping results.

Study design

The following inclusion criteria were followed when collecting the case reports from 2001 through 2012 in the MDSS. A case of STEC was designated under the reportable conditions "*Escherichia coli* O157:H7", "Shiga toxin *E. coli*, unspecified", "Shiga toxin *E. coli*, non-O157", and "Shiga toxin-producing *E. coli* (STEC)". Notably, the reportable conditions of STEC infections in Michigan have undergone updates during the past decade. Before 2010, the three terms "*E. coli* O157:H7", "STEC non-O157", and "STEC unspecified" were used. Beginning in 2010, these three terms were consolidated into one reportable condition called "STEC". Subgrouping of STEC by serotypes was not included under the reportable condition. In addition to the reportable conditions, the case inclusion criteria also included case status, investigation status, and dates of cases reported to the MDCH. Cases were included in the study if case status was "confirmed", the investigation status was "completed", and the report dates were from 01/01/2001 until 12/31/2012.

The demographic, clinical, laboratory, and epidemiologic data were extracted from each case record. Basic demographic information was documented, including age at the onset of disease, gender, race, ethnicity, and date of case was reported. The clinical information included hospitalization, death, HUS status, bloody diarrhea, and non-bloody diarrhea. Presence of other clinical symptoms were also documented, which included fever, chills, abdominal pain, fatigue, body aches, nausea, vomiting, and other health conditions. The laboratory information collected included STEC serotypes and the Shiga toxin (Stx) gene profile. Epidemiologic investigation information included food history (up to seven-day food history), drinking water source (e.g.

municipal, well), animal contact, travel history within one month before the disease onset, and swimming within one month before the disease onset.

Data management and analysis

O157 STEC cases represented all cases with serotype O157 infections, while non-O157 STEC cases were defined as cases infected with all serotypes other than O157. If serotype information was not noted in the case report (stated "unknown"), then the serotype was coded as missing data. The same was true for Shiga toxin type (Stx1 or Stx2) information stated "unknown". Because the symptom onset date or specimen submission date was not available for every case, the "year" and "month" of each case was defined according to the date the case was reported. The season was categorized based on the date of case report: winter (December, January, February), spring (March, April, May), summer (June, July, August), and fall (September, October, November).

Additional data were retrieved from the case reports but were not analyzed further. In general, if the information was not reported in over 50% of the cases, then the information was not included in the analysis. For example, race and ethnicity were "unknown" in 50% of all the cases. Similarly, some clinical symptom data were not analyzed because the definition of the symptoms was unclear in the case reports. The timeline was not available in the reports to distinguish whether the symptoms occurred before the STEC diagnosis or hospitalization, during the hospitalization, or after the STEC diagnosis or discharge from the hospital. These symptoms included fever, chills, abdominal pain, fatigue, body aches, nausea, vomiting, and other health conditions.

The data in the epidemiologic information section were not analyzed further because of the following limitations. First, some of the information was sparse and thus, it was challenging to categorize the information entered as descriptive text (e.g. food history and drinking water source). Second, some of the data were missing in up to 60% of the cases (e.g. animal contact). Lastly, it was difficult to specify the time duration of the potential exposures to the onset of the symptoms (i.e., the potential incubation period). For example, any travel history and swimming information occurred within one month before the disease onset was documented in the case report, but the exact time of the travel and swimming was not available in every case. Therefore, travel history and swimming information could not be accurately evaluated in the analysis.

The annual age-adjusted incidence rates (case per 100,000 population) of STEC cases were computed based on the population estimates of Michigan from the Bridged-Race Population Estimates 1990-2012 dataset (27). The standard population was based on the U.S. 2000 standard population (28, 29). The data management was performed in both Microsoft Excel (Microsoft Corporation, Redmond, WA, U.S.) and SAS 9.3 (SAS Institute, Cary, NC, U.S.).

Chi-square tests were performed to assess the association between STEC O serotypes and the clinical outcomes. The statistical analyses were performed in SAS 9.3. P-values <0.05 were considered to be significant in all statistical tests unless stated otherwise. For assessing the association between characteristics of the reported STEC cases and the clinical outcomes, hospitalization status was used as an indicator for a severe clinical outcome associated with STEC infection. The dependent variable was hospitalization status of the STEC cases (yes: the STEC cases who were hospitalized; no: the STEC cases who were not hospitalized). The variables assessed were STEC serotypes (O157; non-O157), Shiga toxin types (*stx*2-only; *stx*1-only or both *stx*1 and *stx*2), age groups (<5; 5-10; 11-59; >=60), gender (female; male), and

season of report (fall and winter; spring and summer). Univariate analysis was performed, and any independent variable with a p-value <0.25 and considered to be biologically plausible was selected as a candidate for inclusion in a multivariate model using logistic regression. The variable selection method followed the "purposeful selection" steps described by Hosmer and Lemeshow (30). A variable was kept in the model when the p-value was less than 0.1 and removal of this variable caused the values of other variables' effect sizes to change >10%. The univariate analysis and the multivariate analyses were performed using PROC GENMOD function in SAS 9.3.

RESULTS

Descriptive epidemiology of STEC cases

From 2001 to 2012, a total of 1,497 confirmed STEC cases were reported to the MDCH. The highest numbers of STEC cases were reported in 2008 (n=186) and 2012 (n=189), and the lowest was in 2006 (n=88) (Figure 4.1.a). Among these 1,497 STEC cases, 138 (9.2%) cases were confirmed to be associated with outbreaks (n=29). The highest number of outbreak associated cases was 54 in 2012 followed by 47 in 2008. In other years, the outbreak-associated case numbers ranged from 0 between 2001 and 2003 to 12 in 2010. Overall, the age-adjusted incidence rates of STEC cases remained relatively constant before 2007 (ranging from 0.86/100,000 in 2006 to 1.31/100,000 in 2002) (Figure 4.2). The incidence rate peaked for all STEC cases (1.9/100,000) and O157 STEC cases (1.23/100,000) in 2008. No non-O157 STEC cases were reported in 2001. Notably, since 2002, there has been an increasing trend of incidence rate of non-O157 STEC infection. The year 2010 was the first time that incidence of non-O157 STEC cases was greater than O157 STEC cases in Michigan. For all years combined, among O157 STEC cases, the peak in the number of cases (20.4%, 183/895) occurred in September, while non-O157 STEC cases peaked (14.6%, 58/398) in August (Figure 4.3.a.b).

Among all the 1497 cases, STEC O serotypes were not available in 13.6% (203/1497) of the cases. O157 was the most commonly reported serotype among all the STEC cases (69.2%, 895/1497) followed by O45 (11.3%, 146/1497), O103 (5.5%, 71/1497), O26 (4.6%, 60/1497), O111 (2.6%, 33/1497), O145 (1.8%, 23/1497), and O121 (0.8%, 11/1497) (Table 4.1). The remainder of the cases were infected with STEC belonging to 35 additional serotypes. Moreover,

the Shiga toxin gene profiles were available for 1294 (86%) cases. Within the 792 O157 STEC cases with data available, 12 (1.5%) had *stx*1, 284 (35.9%) had *stx*2, and the remaining 496 (63%) had both *stx*1 and *stx*2. The distribution varied among the 399 non-O157 STEC cases as most (n=321; 80%) had *stx*1, followed by *stx*2 (n=46; 12%), and both *stx*1/*stx*2 (n=25; 6%).

Examining the demographic data demonstrated that there were more female STEC cases versus male, which did not vary by serotype, except in O145 cases (Table 4.1). The overall median age in these STEC cases was 22, ranging from 1 day old to 102 years old. The lowest median age by serogroup was 17 years old (0.92-66) in O111 STEC cases, and the highest median age was 27 years old (0.33-85) in cases infected with STEC in O serogroups other than O157, O45, O103, O26, O111, O145, and O121. The age distribution of O157 STEC and non-O157 STEC cases was similar (Figure 4.4.a.b).

Frequencies of clinical outcomes (hospitalization, HUS, bloody diarrhea, and non-bloody diarrhea) in the STEC cases varied by serotype (Table 4.2). Two out of the 1497 (0.1%) cases died within the time of epidemiologic investigation and both were infected with O157 STEC. The proportion of cases hospitalized ranged from 14.1% in O103 STEC cases to 48.2% in O157 STEC cases (Chi-square $p<0.05$). The highest proportion of cases hospitalized was in cases older than 85 years old (100% for both O157 STEC cases and non-O157 STEC cases), and the lowest proportion was in cases younger than 5 years old (7.5% in O157 STEC cases and 25% in non-O157 STEC cases) (Figure 4.5). HUS was reported in 26 (1.7%) out of the 1497 cases, and 22 (84.6%) of the 26 HUS cases were infected with O157 STEC. The proportion of cases that developed bloody diarrhea ranged from 32.7% in STEC cases in non-O157 serotypes other than the six most prevalent non-O157 serotypes to 74.6% in O157 STEC cases (Chi-square $p<0.05$). The proportion of cases with non-bloody diarrhea ranged from 13% in O145 STEC cases to

54.5% in cases infected with STEC in non-O157 serotypes other than the six most prevalent non-O157 serotypes (Chi-square $p<0.05$).

Twenty six cases reported co-infection with more than one STEC serotype or another pathogen; most (n=11; 42.3%) cases were co-infected with *Cryptosporidium* or *Camplybacter* (n=7; 26.9%). One case was co-infected with *Clostridium difficile*, and another was co-infected with *Klebsiella pneumoniae*. One case was co-infected with *Chlamydia* and *Campylobacter*, another was co-infected with *Salmonella*, O157 STEC, and O145 STEC, and another was co-infected with O91:NM STEC and O157 STEC. Cases with co-infections were excluded from the statistical analysis as it was not clear which pathogen contributed to the clinical data highlighted in the MDSS records.

The odds of hospitalization in STEC cases

All together, a total of 880 STEC cases were analyzed to identify covariates associated with hospitalization; 617 cases were excluded from the analysis due to missing data for the outcome variable (hospitalization status) and/or other covariates. All covariates but gender met the initial screening significance level of $p<0.25$ in the univariate analysis (Table 3.3). Among these covariates, the greatest univariate effect sizes were observed in O serotypes and age. The odds of hospitalization were 2.3 times higher in O157 STEC cases when compared with non-O157 STEC cases (95% confidence interval (CI): 1.73, 3.09; $p<0.0001$). Moreover, the odds of hospitalization were in cases aged 60 years or older when compared with cases aged 11 to 59 years (95% CI: 1.79, 3.98; $p<0.0001$).

Because of reporting convention, we elected to force gender in the multivariate analysis. The final model included STEC O serotype (O157; non-O157), age, and gender (Table 4.4). Shiga toxin gene profile and season were removed because they were not significantly associated with hospitalization in the multivariate model. The odds of hospitalization in O157 STEC cases were 2.36 times higher than in non-O157 STEC cases (95% CI:1.76, 3.18; $p<0.0001$). The odds of hospitalization in cases aged 60 years or older old were 2.61 times higher than cases aged 11 to 59 years (95% CI:1.73, 3.93; $p<0.0001$). Cases aged younger than 5 years had odds of hospitalization 0.62 times lower than cases aged 11 to 59 years (95% CI:0.39, 0.98; $p<0.0411$).

DISCUSSION

This study describes STEC cases reported to the MDCH from 2001 through 2012. From both the numbers of cases and the age-adjusted incidence rates, there is a clear increasing trend of non-O157 STEC cases in Michigan over this 12-year period. This increasing trend is likely associated with the improvement of diagnostic tests for non-O157 STEC nationally (17, 31) and in Michigan (20). A peak in the age-adjusted incidence rate was observed among O157 STEC cases in 2008, and this peak may in part be attributed to a large number of outbreak-associated cases (n=47 of a total of 138 outbreak-associated cases) in 2008. The year 2010 was the first time that incidence of non-O157 STEC cases was greater than O157 STEC cases in Michigan. In 2012, the age-adjusted incidence rates were similar between O157 (0.93/100,000) and non-O157 STEC (0.94/100,000) cases. This agrees with the estimation by Scallan *et al*. that more than 50% of total the STEC infections are attributed to non-O157 STEC (8). The CDC have updated the recommendations for detecting non-O157 STEC cases over time (15-17), and the current recommendations are to simultaneously test all stool samples from acute community-acquired diarrhea for STEC O157 by selective and differential media and non-O157 STEC by Shiga toxin assay (17). Testing in parallel or increased diagnostic effort clearly improved sensitivity of detection of STEC infections, and it is essential for the clinical laboratories to continuously follow the current diagnostic recommendations from CDC.

Among all the non-O157 serotypes, O45 was the most reported O serotype, followed by O103, O26, O111, O145, and O121 in Michigan. In the CDC's FoodNet data, O26 was the most commonly reported non-O157 STEC, followed by O103, O111, O121, O45, and O145 (13). Moreover, O111 and O26 were responsible for 66% of the non-O157 STEC outbreaks reported

to the CDC up to 2010 (32). Although the frequency of O serotypes differed in Michigan compared with the FoodNet sites, these six O types predominated in all sites. The differential ranking based on frequency of cases associated by serotype in Michigan may indicate differential risk of exposures regionally in Michigan as compared to FoodNet sites. Other observations in Michigan are consistent with results from FoodNet sites. The demographic characteristic (age and gender) were similar between O157 STEC cases and non-O157 STEC cases in Michigan, and these agree with previous studies in the FoodNet sites (13). However, there were some mild differences. In Michigan, the median age (in years) reported per O serotype ranged from 17 for O111 STEC to 27 for all other non-O157 STEC. This median age range was older than what was reported in the FoodNet reports which, ranged from 9 for O26 to 29 years of age for O121 (13). Although it was difficult to pinpoint factors that contributed to these discrepancies, the number and characteristics of different outbreaks may play a role (13). Future studies are therefore warranted to identify risk factors associated with different non-O157 STEC serotypes. These data may assist in public health interventions through identifying risk factors that may be differential by serotype.

The odds of hospitalization were higher in O157 STEC cases than non-O157 STEC cases in Michigan, which is consistent with data from Minnesota (21) and Connecticut (23). Similarly, one German study has also reported that the risk of hospitalization in O157 STEC cases was equal to or greater than 2 times that in non-O157 STEC cases, except the O104 serotype (33). Interestingly, our model suggested that children younger than 5 years old were associated with lower odds of hospitalization when compared with cases aged 11 to 59 years. The similar age association was reported in the surveillance study in Michigan (20) and one German study (33). This observation suggests other risk factors associated with younger age may affect the risk of

hospitalization, but there is limited information regarding age and odds of hospitalization. Age effect to the risk of STEC infection has been reported elsewhere. In contrast to most reports suggesting similar age distributions between O157 and non-O157 STEC (13), both more likely affecting the young and the old, one study reported that non-O157 STEC infections were more commonly detected in children younger than 5 years old when compared with O157 STEC (24). Age-specific risk factors associated with STEC infections have been reported. For example, eating hamburgers was associated with STEC infections in people aged younger than 12 years old (34). Another study suggested than contact with ruminants was associated with the highest odds of STEC infection in children aged younger than 3 years (35). Whether age associated severity of disease (represented by the proxy measure of hospitalization in this study) is related to differential exposures or a biological component of age warrants further investigation as it may allow for more targeted and cost efficient public health interventions.

The cases included in this study are reported to the MDCH through the public health surveillance system, and they represent a subset of the actual total STEC infections in Michigan. For instance, it is likely that some people infected with STEC developed milder clinical symptoms; and therefore did not seek medical care and were never reported to the public health department (36). Although O157 STEC has been associated with a more severe clinical outcome as suggested by the results of this study, the degree of under-reporting of O157 STEC cases has been estimated to be 20-fold (37). Moreover, the challenges of diagnosing non-O157 STEC in clinical laboratories are widely described (16). A 106.8 fold degree of under-diagnosis was estimated for non-O157 STEC cases, and 26.1 fold of which for O157 STEC cases (8). As a result, the cases reported to the MDCH are an underestimated number of actual STEC incidence in Michigan due to the effect of under-reporting or under-diagnosis. In addition, public health

surveillance systems are not designed for research, but for monitoring trend of diseases, identifying outbreaks and assessing the impact of public health prevention and control measures to disease frequencies (22, 36, 38). Therefore, there are inherent limitations when it comes to the use of surveillance data for risk factor analysis. For example, some data have more than 50% of missing values, e.g. animal contact information. Despite these limitations, reviewing surveillance cases may reveal at-risk populations as these cases may be valuable for understanding STEC cases with more severe clinical outcomes or individuals with higher risk of infection. They are certainly a biased representation of all STEC cases and therefore likely overestimate the magnitude of effect of the risk factors evaluated in this study.

Notably, non-O157 STEC contains STEC in heterogeneous serotypes. Some reports have suggested that STEC in different serotypes have different epidemiologic characteristics. For example, O111, O103 and O26 were more common among cases reporting history of international travel (13, 23). Treating a heterogeneous group of bacteria (non-O157 STEC) as a single serotype category limits our abilities to fully understand risk factors for disease, and therefore may prevent more effective interventions. Future studies are needed to examine the epidemiologic characteristics in different O serotypes, for example, the age effect to the severity of illness in different O types or differential risk of exposure to certain serotypes geographically in Michigan.

Whether increasing in actual incidence or through an increase in our ability to diagnose cases, non-O157 STEC, a heterogeneous and not fully understood group of pathogens, represent a significant public health concern in Michigan (as in the U.S.) and is not only in disease estimates but in cases reported likely to surpass O157:H7. Although non-O157 STEC were less likely to result in hospitalization, the increasing incidence of this heterogeneous group of

pathogens demonstrates a significant public health cost. Investment in research to more fully elucidate risk factors for STEC infection, including serotype and more complete human risk factor data, are needed.

ACKNOWLEDGEMENTS

Sincere thanks to Dr. James Rudrik, James Collins and Tiffany Henderson of the Michigan Department of Community Health for assisting with collection of epidemiologic and laboratory data as well as the National Institues of Health Enterics Research Investigational Network (ERIN) Cooperative Research Center at Michigan State University [grant number U19AI090872 (SDM)] and in part by the United State Department of Agriculture, National Institute of Food and Agriculture [grant number 2011-67005-30004(SMD)].

APPENDIX

Table 4.1. Demographic characteristics of STEC cases by STEC O serotypes, Michigan, 2001-2012

		O serotypes[b]							
	Total no. of cases (%)	O157 (n=895, 69.2%[c])	O45 (n=146, 11.3%)	O103 (n=71, 5.5%)	O26 (n=60, 4.6%)	O111 (n=33, 2.6%)	O145 (n=23, 1.8%)	O121 (n=11, 0.8%)	Others[d] (n=55, 4.3)
Age (year)[a]									
<5	181(12.1)	108(12[d])	13(8.9)	12(16.9)	6(1)	9(27.3)	0	0	8(14.5)
5-10	161(10.8)	102(11.3)	14(9.6)	5(7)	5(8.3)	4(12.1)	1(4.3)	3(27.3)	6(10.9)
11-69	1050(70.1)	627(70.1)	110(75.3)	52(73.2)	46(76.7)	20(60.6)	20(87)	8(72.7)	34(61.8)
>70	105(7)	58(6.5)	9(6.2)	2(2.8)	3(5)	0	2(8.7)	0	7(12.7)
Gender[a]									
Male	691(46.5)	421(47)	61(41.8)	33(46.5)	21(35)	15(45.5)	13(56.6)	3(27.3)	26(42.3)
Female	795(53.5)	469(52.4)	85(58.2)	38(53.5)	38(63.3)	16(48.5)	10(43.5)	8(72.7)	28(50.9)

[a]Gender was not available in n=11 cases.

[b]Serotype was not available in 13.6% (203/1497) cases. O157 includes O157, O157:H7, O157:NM (non motile), O157:H rough, O157:H undetermined, O157:H unknown. O45 in cludes O45:H undetermined, O45:H2. O103 includes O103, O103:H11, O103:H2, O103:H25. O26 includes O26:H11, O26:NM. O111 includes O111, O111:H8, O111:NM. O145 includes O145, O145:NM. O121

Table 4.1. (cont'd)

includes O121, O121:H19, O121:H7, O121:H9. Others include the following serotypes: O rough:H2, O rough:NM, O undertermined:H undertermined, O undertermined:H11, O undertermined:H19, O undetermine:NM, O undetermined:H11, O undetermined:H25, O undetermined:H52, O undetermined:NM ,O104:H4, O123:H11, O128:H25, O128:NM, O123:H11,O128:NM, O147:H7, O156:H25, O156:NM, O163:H19, O165:NM, O168:H8, O174:H21, O177:NM, O185:H28, O186:H11,O186:H2, O39:H49, O49:NM, O5:NM, O69:H11, O71:H11, O71:NM, O76:H19, O91:NM, two cases with two STEC strains isolated (one with O157, O145; one with O91:NM, O157).

[c]% represents the % of total no. of cases with O serogroup information (n=1294)

[d]% represents the % of total no. of cases within each O serogroup.

Table 4.2. Clinical outcomes of STEC cases by STEC O serotypes, Michigan, 2001-2012

		Hospitalization	HUS	Bloody diarrhea	Non-bloody diarrhea
O serotypes[a]	Total	No. of cases (%[b])	No. of cases (%)	No. of cases (%)	No. of cases (%)
O157	895	432(48.2)	22(2.5)	668(74.6)	145(16.2)
O45	146	60(41.1)	0(0)	94(64.3)	42(28.7)
O103	71	10(14.1)	1(1.4)	31(43.6)	31(43.7)
O26	60	13(21.6)	0	34(56.7)	20(33.3)
O111	33	12(36.3)	0	13(39.4)	11(33.3)
O145	23	7(30.4)	0	19(82.6)	3(13)
O121	11	5(45.5)	0	6(54.5)	4(36.4)
Others	55	18(32.7)	1(1.8)	18(32.7)	30(54.5)
Unknown	203	54(26.6)	2(1)	67(33)	44(21.6)
Total	1497	611(40.8)	26(1.7)	956(63.9)	330(22)

[a] Serotype information was not available in 13.6% (203/1497) cases (O serogroup "unknown"). O157 includes O157, O157:H7, O157:NM (non motile), O157:H rough, O157:H undetermined, O157:H unknown. O45 in cludes O45:H undetermined, O45:H2. O103 includes O103, O103:H11, O103:H2, O103:H25. O26 includes O26:H11, O26:NM. O111 includes O111, O111:H8, O111:NM.

Table 4.2. (cont'd)

O145 includes O145, O145:NM. O121 includes O121, O121:H19, O121:H7, O121:H9. Others include the following serotypes: O rough:H2, O rough:NM, O undertermined:H undertermined, O undertermined:H11, O undertermined:H19, O undetermine:NM, O undetermined:H11, O undetermined:H25, O undetermined:H52, O undetermined:NM ,O104:H4, O123:H11, O128:H25, O128:NM, O123:H11,O128:NM, O147:H7, O156:H25, O156:NM, O163:H19, O165:NM, O168:H8, O174:H21, O177:NM, O185:H28, O186:H11,O186:H2, O39:H49, O49:NM, O5:NM, O69:H11, O71:H11, O71:NM, O76:H19, O91:NM, two cases with two STEC strains isolated (one with O157, O145; one with O91:NM, O157).

[b]% of total cases within each O serogroup.

[c]Hospitalization status was missing in 9.9% (148/1497) case reports. HUS status was stated "unknown" in 10.8% (162/1497) case reports. Death status was stated "unknown" in 0.5% (8/1497) case reports. Bloody diarrhea and diarrhea status was stated "unknown" in 11% (165/1497) case reports.

Table 4.3. Univariate analysis of characteristics associated with hospitalization in STEC cases, Michigan, 2001-2012

Characteristic	No. with characteristic	No (%) hospitalized	OR[a]	95% CI[b]	p-value[c]
O serogroup					
O157	574	299(52.1)	2.3	(1.73-3.09)	<0.0001
Non-O157 (reference)	306	98(32)			
Shiga toxin profiles					
*stx*2-only	248	131(52.8)	1.5	(1.15-2.07)	0.0041
*stx*1-only, *stx*1 and *stx*2 (reference)	632	266(42.1)			

Table 4.3. (cont'd)

Characteristic	No. with characteristic	No. (%) hospitalized	OR[a]	95% CI[b]	p-value[c]
Age groups					
<5	97	32(33)	0.64	(0.49-1.2)	0.247
5-10	97	36(37.1)	0.77	(0.41-1.01)	0.056
11-59 (reference)	555	241(43.4)			
>=60	131	88 (67.2)	2.67	(1.79-3.98)	<0.0001
Gender					
Female	475	222(46.7)	1.15	(0.883-1.506)	0.2948
Male (reference)	405	175(43.2)			

Table 4.3. (cont'd)

Characteristic	No. with characteristic	No. (%) hospitalized	OR[a]	95% CI[b]	p-value[c]
Season					
Fall and winter	422	203(48.1)	1.26	(0.97-1.65)	0.0872
Spring and summer (reference)	458	194(42.4)			

[a]Odds ratio

[b]95% confidence interval

[c] Walds Chi-square test

Table 4.4. Multivariate logistic regression model for characteristics associated with hospitalization in STEC cases, Michigan, 2001-2012

Parameter	Regression coefficient	Standard error	OR[a]	95% CI[b]	p-value[c]
Intercept	-0.8798	0.1566			<0.0001
O157 (reference: non-O157)	0.8601	0.152	2.36	(1.76-3.18)	<0.0001
Female (reference: male)	0.1093	0.152	1.12	(0.84-1.47)	0.4423
Age <5	-0.4838	0.2368	0.62	(0.39-0.98)	0.0411
Age 5-10	-0.3425	0.2325	0.71	(0.45-1.12)	0.1407
Age >60	0.9602	0.2098	2.61	(1.73-3.93)	<0.0001
(Age reference: 11-59)					

[a]Odds ratio

[b]95% confidence interval

[c] Walds Chi-square test

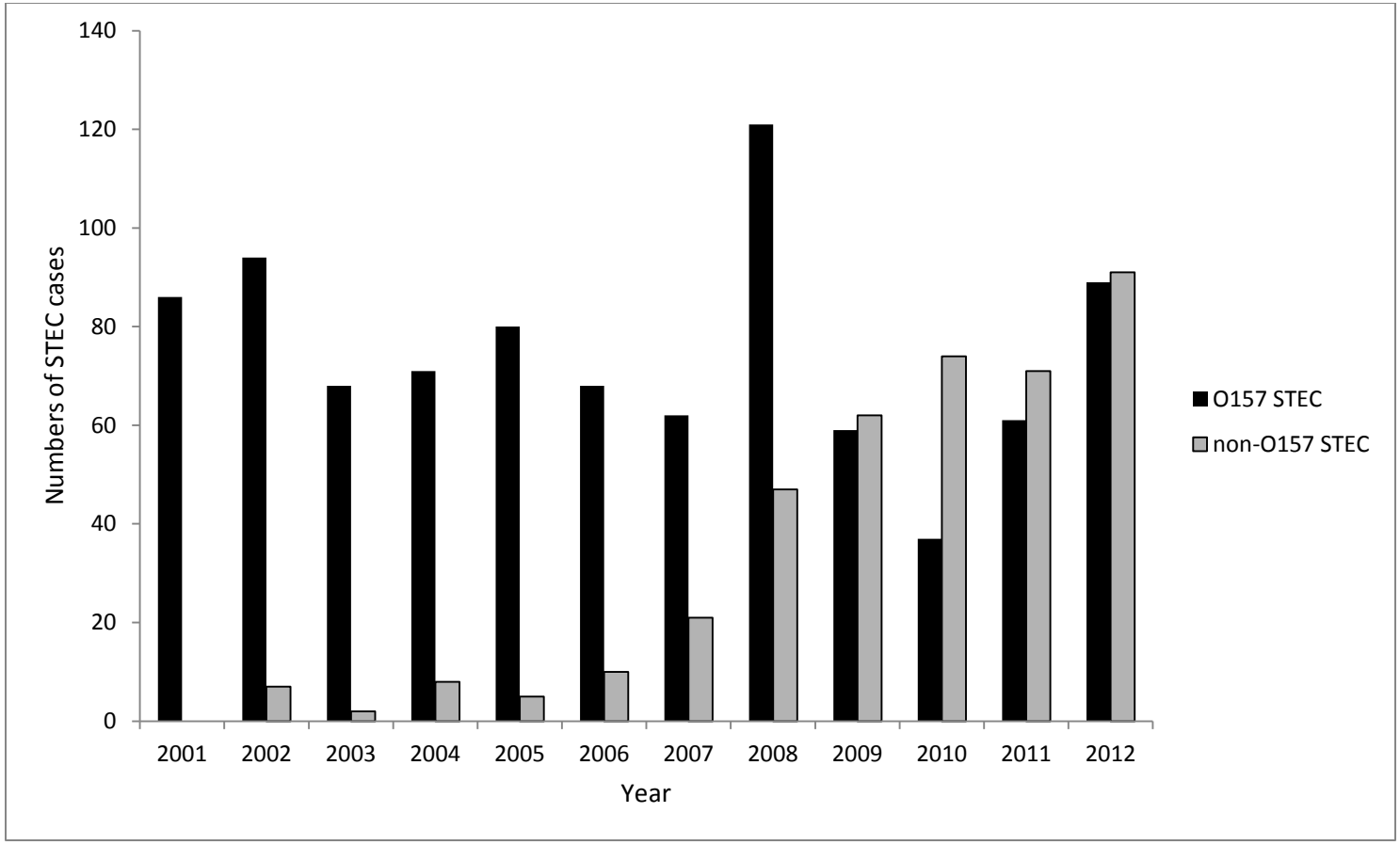
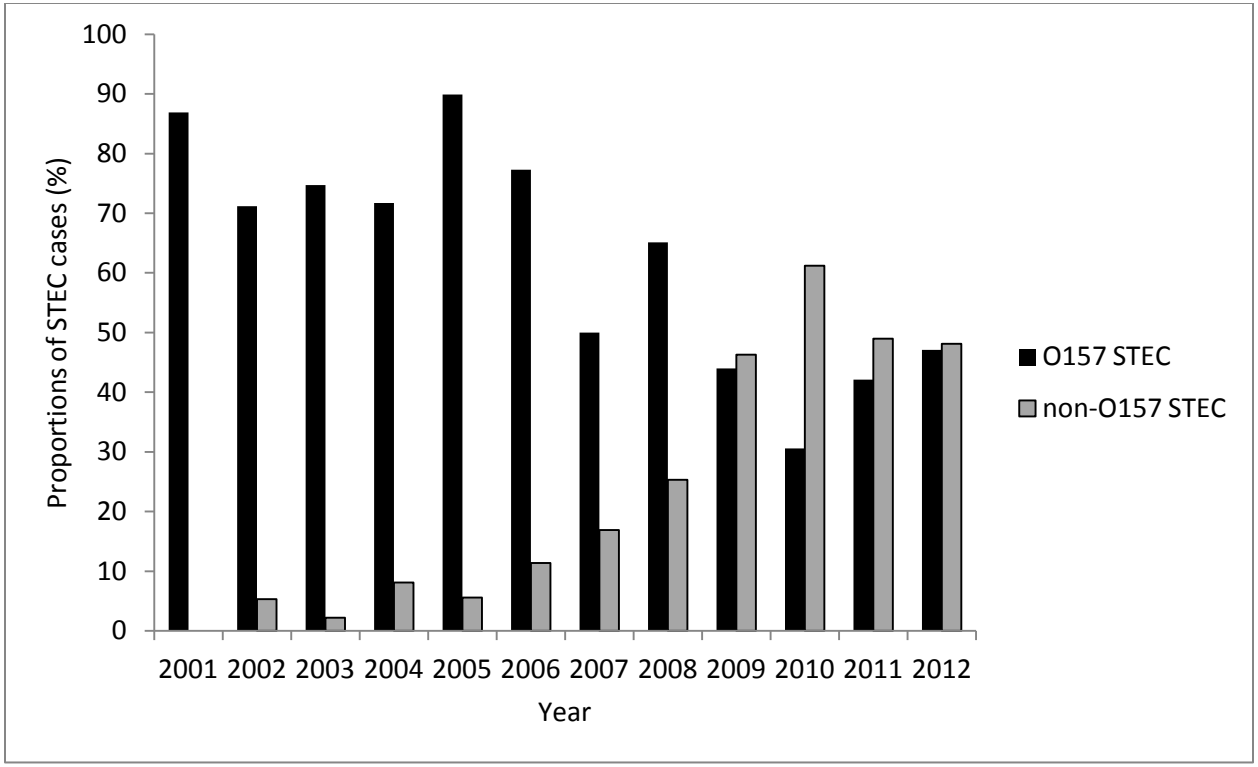


Figure 4.1.a. Numbers of O157 STEC cases and non-O157 STEC cases, Michigan, 2001-2012 (representing total n=1294; excluding n=203 with no serotype information)



[a]Proportions of O157 (non-O157) STEC cases out of all STEC cases reported in each year

Figure 4.1.b. Proportions[a] of O157 STEC cases and non-O157 STEC cases, Michigan, 2001-2012 (representing total n=1294; excluding n=203 with no serotype information)

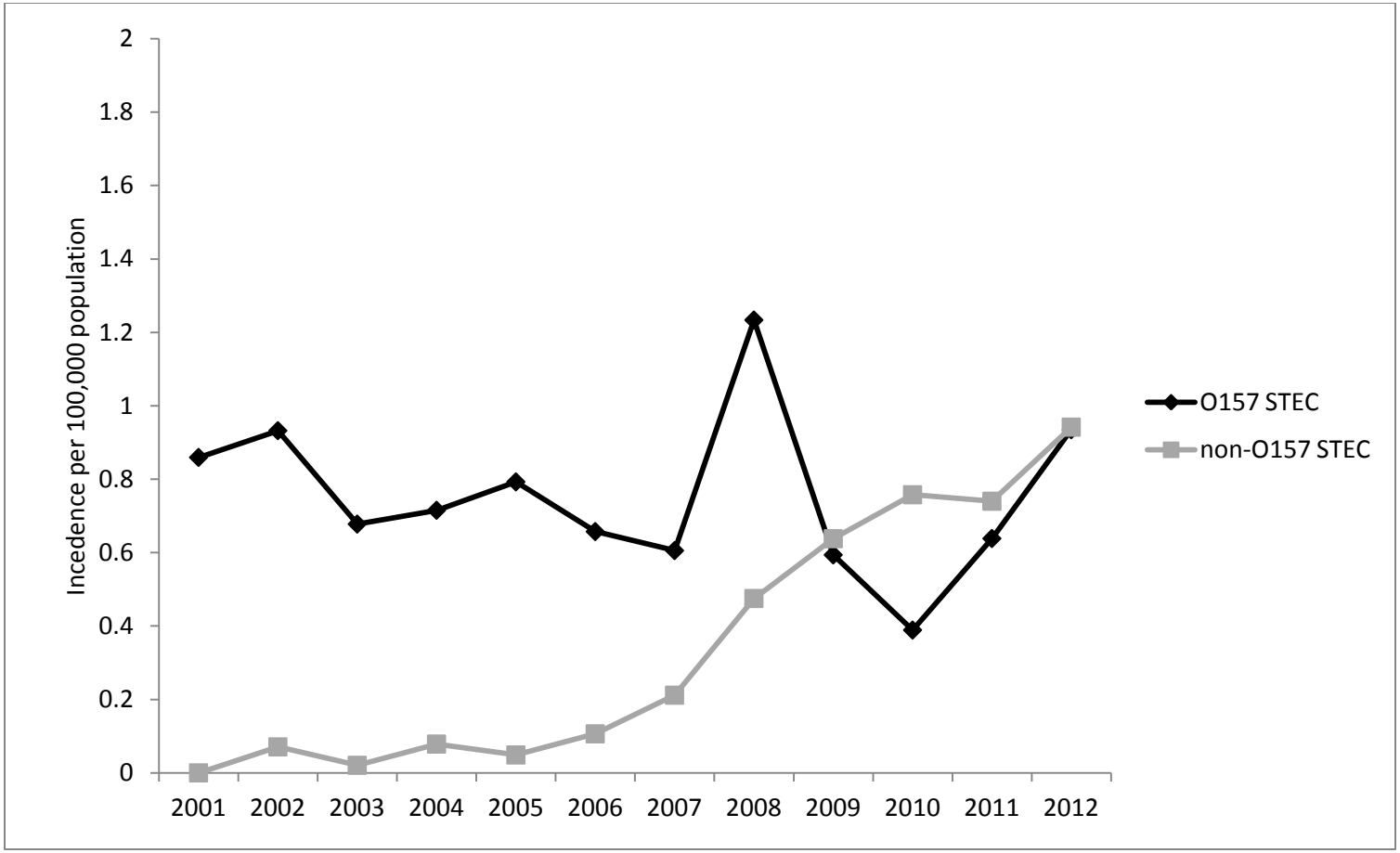


Figure 4.2. Age-adjusted incidence rates of STEC cases per 100,000 population by year, Michigan, 2001-2012 (representing total n=1294; excluding n=203 with no serotype information)

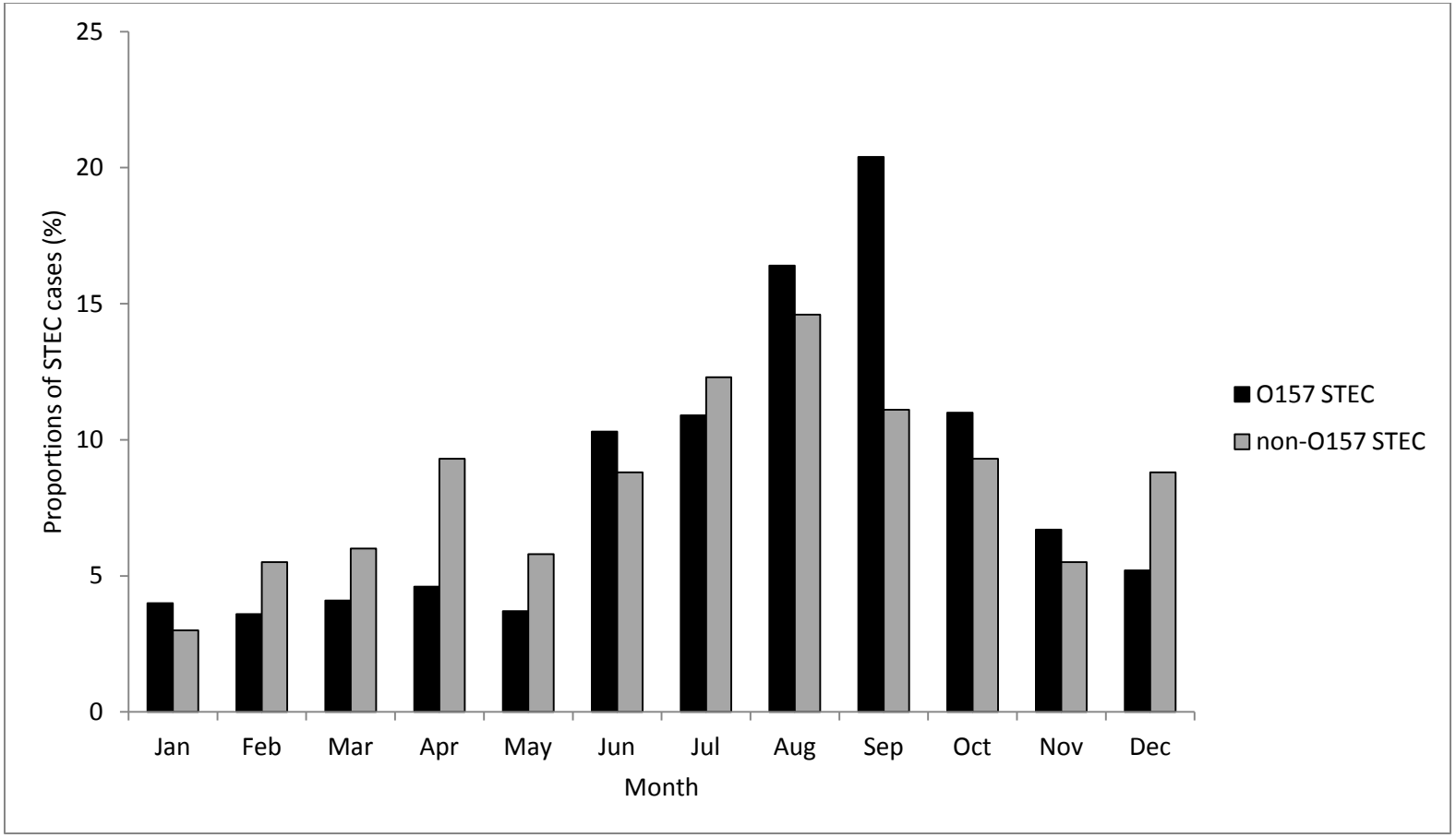
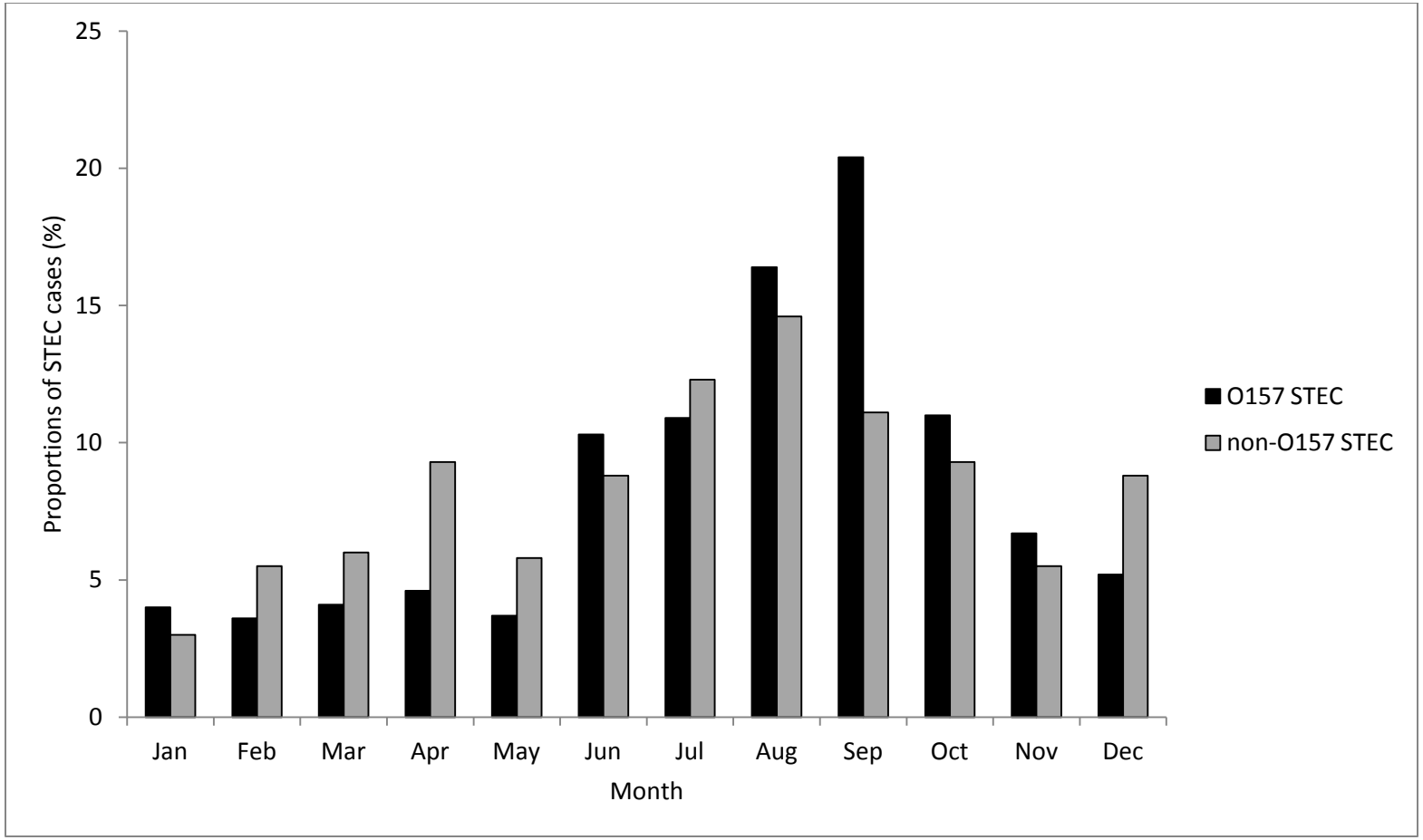


Figure 4.3.a. Numbers of STEC cases by month of report, Michigan, 2001-2012 (representing total n=1294; excluding n=203 with no serotype information)



[a]Proportions of O157 (non-O157) STEC reported in each month out of all O157 STEC (non-O157) cases 2001-2012

Figure 4.3.b. Proportions[a] of STEC cases by month of report, Michigan, 2001-2012 (representing total n=1294; excluding n=203 with no serotype information)

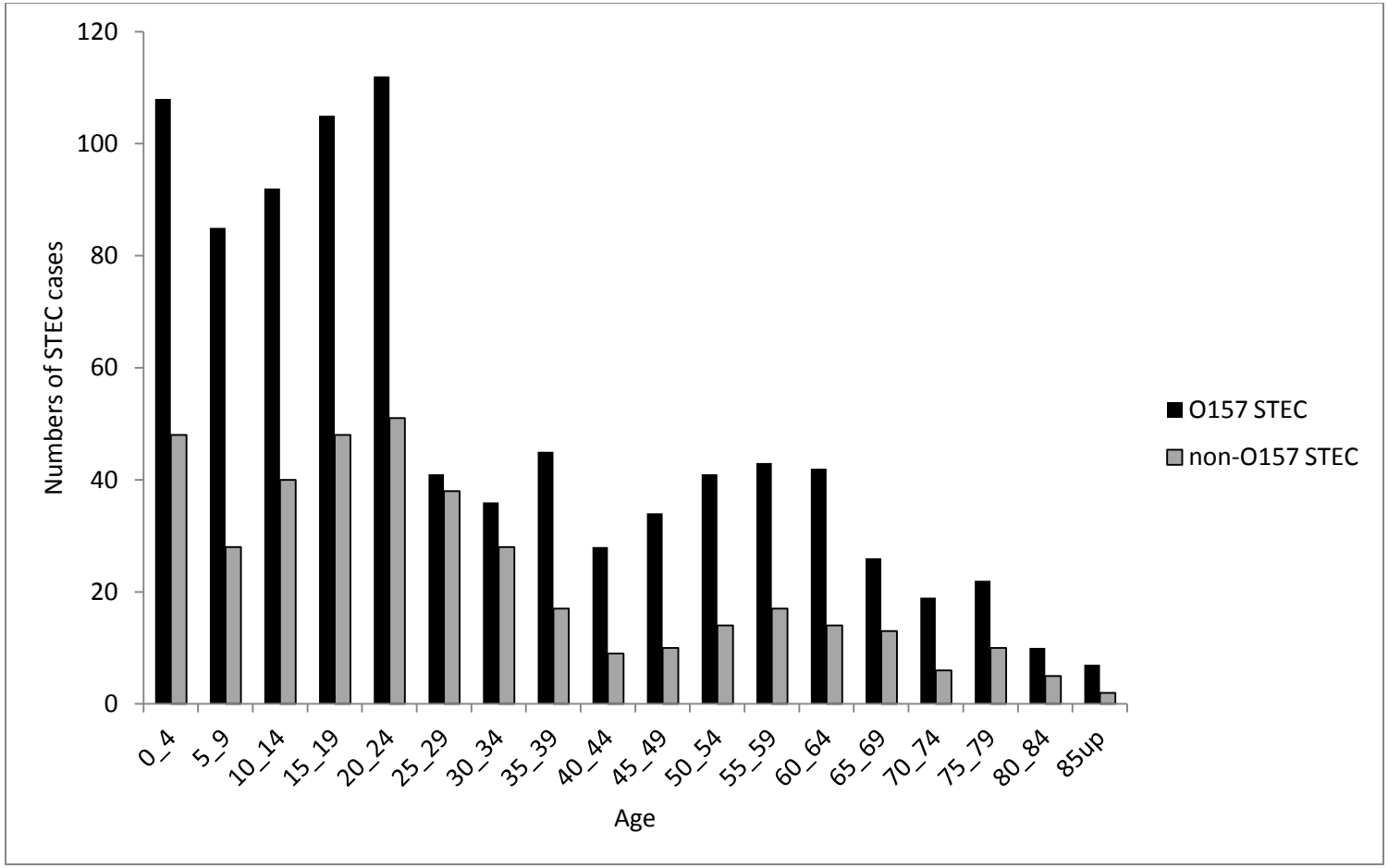
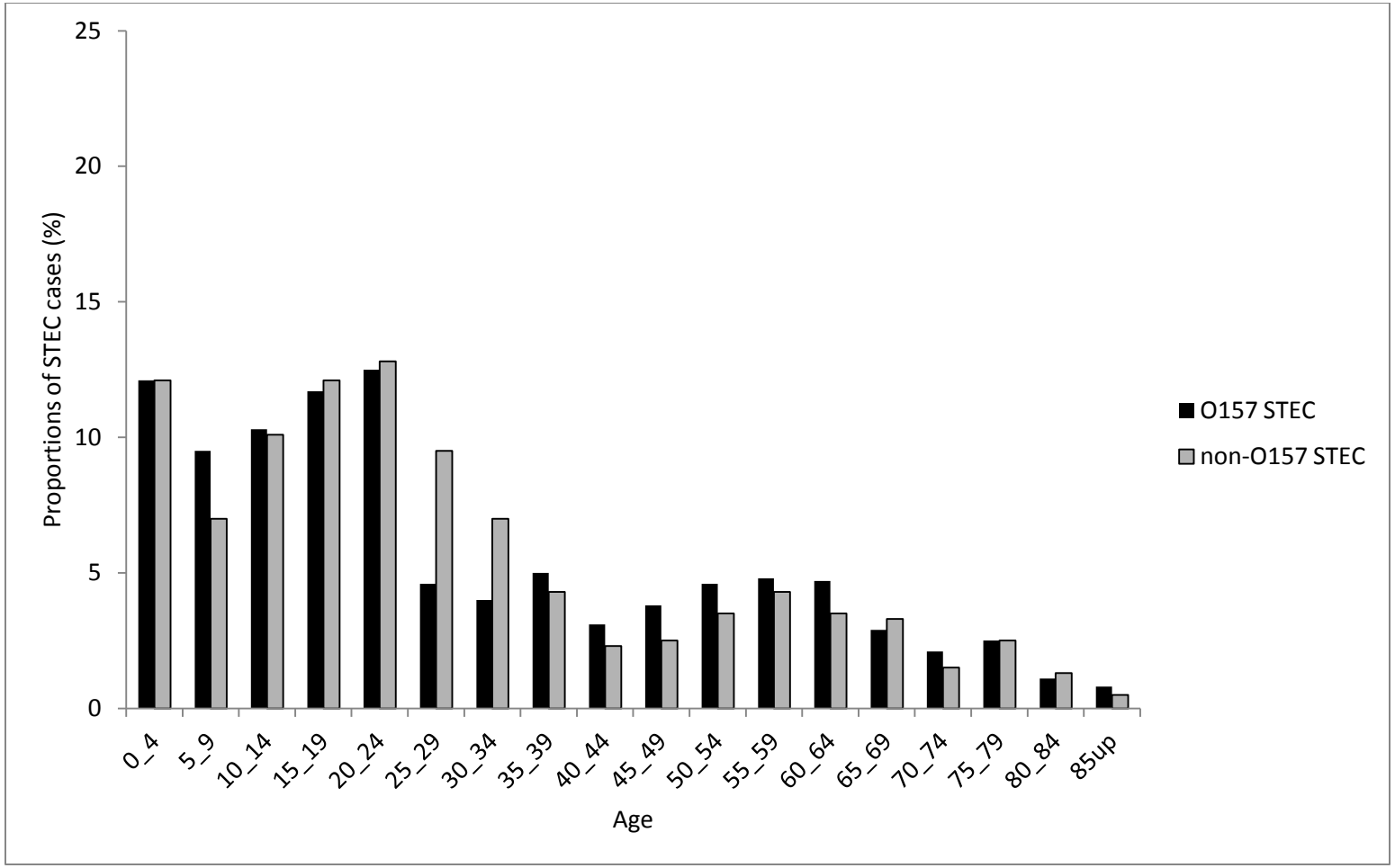


Figure 4.4.a. Numbers of O157 STEC cases and non-O157 STEC by age groups, Michigan, 2001-2012 (representing total n=1294; excluding n=203 with no serotype information)



[a]Proportions of numbers of O157 STEC (non-O157) cases in each age group out of all O157 STEC (non-O157) cases.

Figure 4.4.b. Proportions[a] of O157 STEC cases and non-O157 STEC by age groups, Michigan, 2001-2012 (representing total n=1294; excluding n=203 with no serotype information)

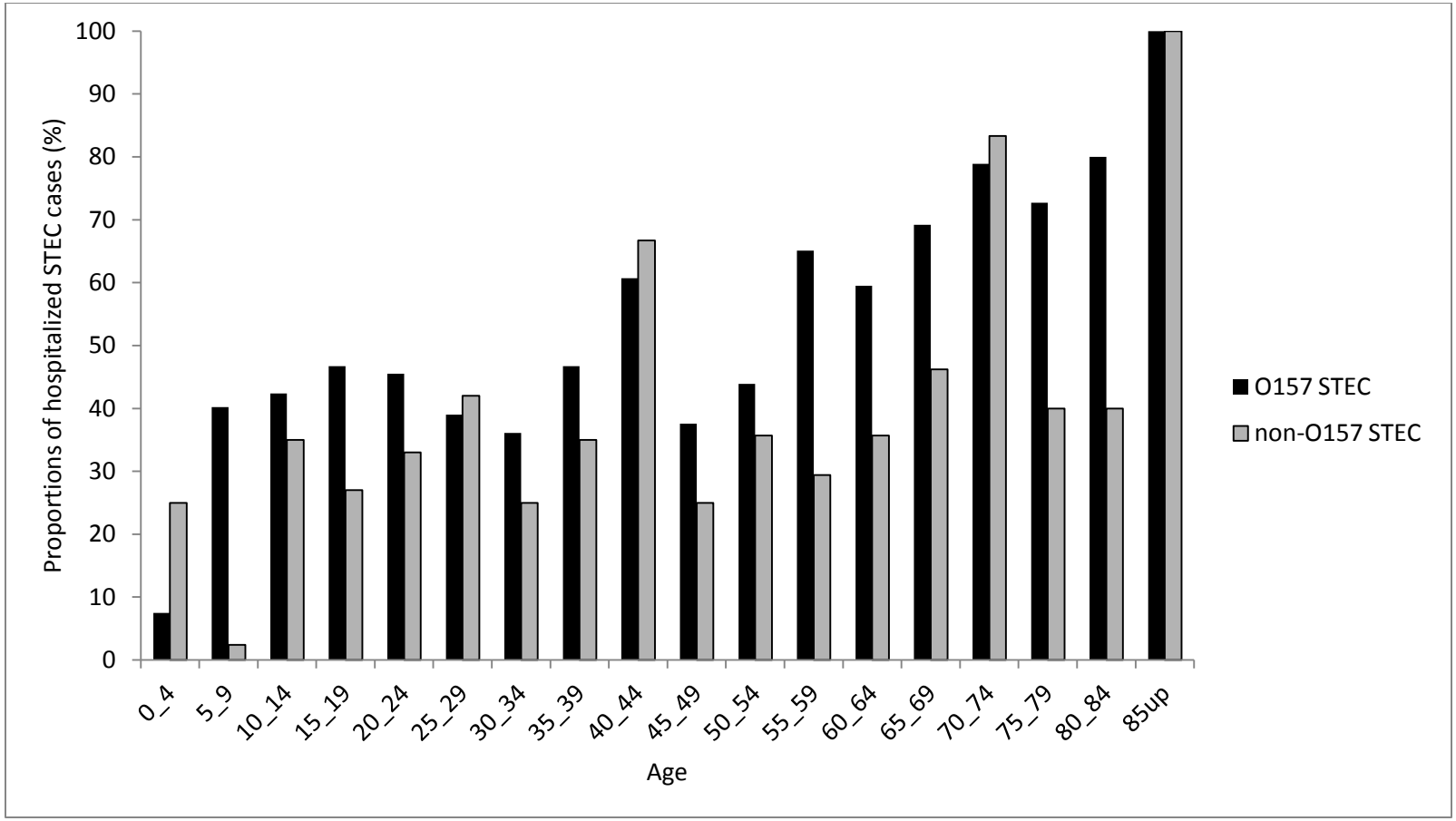


Figure 4.5. Proportion of hospitalized STEC cases by age group and serotype, Michigan, 2001-2012 (representing total n=1294; excluding n=203 with no serotype information)

REFERENCES

REFERENCES

1. Nataro, J.P. and J.B. Kaper, *Diarrheagenic Escherichia coli.* Clin Microbiol Rev, 1998. **11**(1): p. 142-201.

2. Karmali, M.A., et al., *The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing Escherichia coli.* J Infect Dis, 1985. **151**(5): p. 775-82.

3. Tarr, P.I., C.A. Gordon, and W.L. Chandler, *Shiga-toxin-producing Escherichia coli and haemolytic uraemic syndrome.* The Lancet, 2005. **365**(9464): p. 1073-1086.

4. Zoja, C., S. Buelli, and M. Morigi, *Shiga toxin-associated hemolytic uremic syndrome: pathophysiology of endothelial dysfunction.* Pediatr Nephrol, 2010. **25**(11): p. 2231-40.

5. Noris, M. and G. Remuzzi, *Hemolytic uremic syndrome.* J Am Soc Nephrol, 2005. **16**(4): p. 1035-50.

6. Karmali, M.A., V. Gannon, and J.M. Sargeant, *Verocytotoxin-producing Escherichia coli (VTEC).* Vet Microbiol, 2010. **140**(3-4): p. 360-70.

7. Williams, D.M., et al., *Acute kidney failure: a pediatric experience over 20 years.* Arch Pediatr Adolesc Med, 2002. **156**(9): p. 893-900.

8. Scallan, E., et al., *Foodborne Illness Acquired in the United States—Major Pathogens.* Emerging Infectious Diseases, 2011. **17**(1): p. 7-15.

9. Gyles, C.L., *Shiga toxin-producing Escherichia coli: an overview.* J Anim Sci, 2007. **85**(13 Suppl): p. E45-62.

10. Henderson, H., *Direct and indirect zoonotic transmission of Shiga toxin-producing Escherichia coli.* Journal of the American Veterinary Medical Assocciation, 2008. **232**(6): p. 848-59.

11. Law, D., *Virulence factors of Escherichia coli O157 and other Shiga toxin-producing E. coli.* J Appl Microbiol, 2000. **88**(5): p. 729-45.

12. Bettelheim, K.A., *The non-O157 Shiga-toxigenic (verocytotoxigenic) Escherichia coli; under-rated pathogens.* Crit Rev Microbiol, 2007. **33**(1): p. 67-87.

13. Gould, L.H., et al., *Increased recognition of non-O157 Shiga toxin-producing Escherichia coli infections in the United States during 2000-2010: epidemiologic features and comparison with E. coli O157 infections.* Foodborne Pathog Dis, 2013. **10**(5): p. 453-60.

14. FSIS, *Food Safety and Inspection Service. Shiga toxin-producing Escherichia coli in certain raw beef products.* Federal Register, 2011. **76**: p. 58157-58165.

15. CDC, *Importance of culture confirmation of Shiga toxin-producing Escherichia coli infection as illustrated by outbreaks of gastroenteritis---New York and North Carolina, 2005.* Morbidity and Mortality Weekly Report, 2006. **55**(38): p. 1042-1045.

16. CDC, *Recommendations for diagnosis of Shiga toxin-producing Escherichia coli infections by clinical laboratories.* Morbidity and Mortality Weekly Report, 2009. **58**(RR-12): p. 1-14.

17. Gould, L.H., *Update: Recommendations for Diagnosis of Shiga Toxin-Producing Escherichia coli Infections by Clinical Laboratories.* Clinical Microbiology Newsletter, 2012. **34**(10): p. 75-83.

18. Hoefer, D., et al., *Laboratory practices for the identification of Shiga toxin-producing Escherichia coli in the United States, FoodNet sites, 2007.* Foodborne Pathog Dis, 2011. **8**(4): p. 555-60.

19. Stigi, K.A., et al., *Laboratory practices and incidence of non-O157 shiga toxin-producing Escherichia coli infections.* Emerging Infectious Diseases, 2012. **18**(3): p. 477-9.

20. Manning, S.D., et al., *Surveillance for Shiga toxin-producing Escherichia coli, Michigan, 2001-2005.* Emerging Infectious Diseases, 2007. **13**(2): p. 318-21.

21. Hedican, Erin B., et al., *Characteristics of O157 versus Non-O157 Shiga Toxin–ProducingEscherichia coliInfections in Minnesota, 2000–2006.* Clinical Infectious Diseases, 2009. **49**(3): p. 358-364.

22. Tauxe, R.V., *Real burden and potential risks from foodborne infections: the value of multi-jurisdictional collaborations.* Trends in Food Science & Technology, 2008. **19**: p. S18-S25.

23. Hadler, J.L., et al., *Ten-year trends and risk factors for non-O157 Shiga toxin-producing Escherichia coli found through Shiga toxin testing, Connecticut, 2000-2009.* Clinical Infectious Diseases, 2011. **53**(3): p. 269-76.

24. Lathrop, S., K. Edge, and J. Bareta, *Shiga toxin-producing Escherichia coli, New Mexico, USA, 2004-2007.* Emerging Infectious Diseases, 2009. **15**(8): p. 1289-91.

25. MDCH. [cited 2013, 2014; Available from: http://www.michigan.gov/mdch/0,4612,7-132-2945_5104_31274---,00.html.

26. MDCH (2014) *Reportable diseases in Michigan-2014*.

27. United States Department of Health and Human Services (US DHHS), C.f.D.C.a.P. and N.C.f.H.S.N. (CDC). *Bridged-Race Population Estimates, United States July 1st resident population by state, county, age, sex, bridged-race, and Hispanic origin.* 2013 13 June 2013 [cited 2014 06 March]; Compiled from 1990-1999 bridged-race intercensal population estimates (released by NCHS on 7/26/2004); revised bridged-race 2000-2009 intercensal population estimates (released by NCHS on 10/26/2012); and bridged-race Vintage 2012 (2010-2012) postcensal population estimates (released by NCHS on 6/13/2013). :[Available from: http://wonder.cdc.gov/bridged-race-v2012.html

28. Anderson, R.N. and H.M. Rosenberg, *Age standardization of death rates: implementation of the year 2000 standard.* National Vital Statistics Reports, 1998. **47**(3): p. 1-16, 20.

29. Klein RJ, S.C., *Age Adjustment Using the 2000 Projected U.S. Population.* . 2001, National Center for Health Statistics: Hyattsville, Maryland.

30. David W. Hosmer, S.L., *Model-building strategies and methods for logistic regression*, in *Applied Logistic Regression* 2000, John Wiley & Sons, Inc: Hoboken, NJ. p. 91-142.

31. CDC (2013) *National Shiga toxin-producing Escherichia coli (STEC) surveillance annual report, 2011*.

32. Luna-Gierke, R.E., et al., *Outbreaks of non-O157 Shiga toxin-producing Escherichia coli infection: USA.* Epidemiology and Infection, 2014: p. 1-11.

33. Preussel, K., et al., *Shiga toxin-producing Escherichia coli O157 is more likely to lead to hospitalization and death than non-O157 serogroups--except O104.* PLoS One, 2013. **8**(11): p. e78180.

34. Voetsch, A.C., et al., *Risk factors for sporadic Shiga toxin-producing Escherichia coli O157 infections in FoodNet sites, 1999-2000.* Epidemiology and Infection, 2007. **135**(6): p. 993-1000.

35. Werber, D., et al., *Shiga toxin-producing Escherichia coli infection in Germany: different risk factors for different age groups.* American Journal of Epidemiology, 2007. **165**(4): p. 425-34.

36. Tauxe, R.V., et al., *Evolving public health approaches to the global challenge of foodborne infections.* International Journal of Food Microbiology, 2010. **139 Suppl 1**: p. S16-28.

37. Mead, P.S., et al., *Food-related illness and death in the United States.* Emerging Infectious Diseases, 1999. **5**(5): p. 607-25.

38. Crim, S.M., et al., *Incidence and trends of infection with pathogens transmitted commonly through food - foodborne diseases active surveillance network, 10 u.s. Sites, 2006-2013.* MMWR. Morbidity and Mortality Weekly Report, 2014. **63**(15): p. 328-32.