RESEARCH REPORT
PORK SAFETY

Title: Epidemiology of STEC in Swine, #10-128

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Industry Summary:
Shiga-toxin producing *Escherichia coli* (STEC) are important foodborne pathogens. Although most STEC infections in humans are attributed to beef products, outbreaks of STEC have been associated with pork products, and STEC have been isolated from swine and pork products. There is little information regarding the epidemiology of STEC on swine farms. The objectives of this project were to 1) describe the epidemiology of STEC in swine; 2) Characterize these STEC isolates and 3) Develop an infectious disease model of STEC transmission. A longitudinal study was conducted in one swine production company in the Midwest. Three separate groups of finisher pigs (10 -26 weeks of age) were included in the study. In each cohort, 50 pigs were randomly selected and individually identified with an ear tag (3 cohorts X 50 pigs = 150 total pigs). Fecal samples were collected every 2 weeks during the finishing period for a total of 8 samples per pig. STEC isolates recovered from fecal culture were serotyped (O type) and assayed for the presence or absence of the *eae* gene, as well as the shiga toxin gene subtype (*stx* gene). Ninety-eight of 150 pigs (65.3%) of pigs were detected as shedding STEC at least once during the finishing phase. The proportion of pigs positive at any one time point in any cohort ranged from 0 to 62%. The number of collections a pig was STEC positive ranged from 1 to 4 times. No STEC isolates were detected to harbor the intimin gene (*eae*). Nine different serogroups were identified, with O serogroup O59 being the most common (80.9% of isolates). Most isolates harbored the stx_{2e} subtype of shiga-toxin. These results indicate that, at least on this farm, STEC is shed at relatively high incidence in finishing pigs, at rates similar to that reported in cattle. No isolates harbored the intimin gene, which is associated with severe disease in humans. Initial development of a susceptible-infected-susceptible model of STEC transmission in pigs suggest the potential for persistence of infection over time. More data on STEC shedding in swine is needed to fully understand transmission dynamics. These results provide the key initial data for understanding the epidemiology of STEC and determination of the foodborne hazard associated with STEC in swine.

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Scientific Abstract:

Shiga toxin-producing *Escherichia coli* (STEC) are important public health concern, causing more than 200,000 cases of illness annually in the United States. STEC infections are associated with severe clinical diseases in humans: hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The majority of STEC infections are attributed to food or water contaminated by animal feces. There have been outbreaks and cases of STEC infections in humans associated with pork products. However, little is known about swine STEC to date. We have conducted a descriptive longitudinal study to achieve our objectives: to describe the epidemiology of STEC shedding in US swine during the finishing period and to characterize swine STEC strains. **Methods:** This study included three cohorts of pigs from one production company. In each cohort, 50 randomly-selected pigs were individually identified and followed through the finishing period. Fecal samples were collected from each pig every two weeks within the 16 weeks of the finishing period (eight samples/pig). Samples were submitted for STEC detection by enrichment (10 min in TSB, pH 3 followed by incubation for 15 h in modified TSB, pH 8.7 at 41 °C) followed by the polymerase chain reaction (PCR) targeting the Shiga toxin genes (*stx*) and the intimin protein gene (*eae*). Shiga toxin gene-positive samples were plated onto ChromAgar STEC. Presumptive STEC isolates were recovered and confirmed. Serotypes and *stx* gene subtypes were characterized. **Results:** In general, STEC was detected in samples from 31 out of the 50 pigs in cohort 1, 27 out of the 50 pigs in cohort 2, and 40 out of the 50 pigs in cohort 3. STEC was detected more than one time in over 50 % of the pigs in each cohort. The shedding patterns within the finishing period were similar to outbreak curves. No *eae* gene has been detected in these swine STEC strains.
Conclusions:
These data will be critical to fill the current knowledge gaps in swine STEC epidemiology and the association of swine STEC and public health.

Introduction:

Failures in food safety systems remain a major public health threat. Despite significant efforts, near term public health goals for reduction in foodborne illness will not be met (Anonymous, 2009). As demonstrated by recent large-scale foodborne outbreaks, failures in the process can have significant and far-reaching public health and economic impacts.

Human illness associated with shiga-toxin producing Escherichia coli (STEC) are not excluded from the lack of progress in decrease of human illness. Cattle are well known as the primary reservoir for human infection STEC in the USA. The epidemiology of STEC shedding in cattle and its zoonotic food-borne transmission has been well characterized (Hussein and Sakuma, 2005; Gyles, 2007). Other species, including swine have been reported to shed STEC (O157 and non-O157) (Bettleheim, 2007). Both in the US and in other countries, pigs have been found to harbor STEC at prevalence rates similar to those reported in cattle, and with strains that can potentially cause human illness (Borie et al, 1997; DesRosiers et al, 2001; Nazakawa and Akiba, 1998; Johnsen et al, 2001; Feder et al, 2003; Rios et al, 1999, Fratamico et al, 2004; Bettleheim, 2007). Additionally, STEC have been isolated from pork products, and foodborne illness associated with consumption of STEC contaminated pork products has been reported (Bettleheim, 2007; Conedera et al, 2007). There is a paucity of data regarding the epidemiology of STEC in swine, and limited data regarding the association between pigs, pork and human illness.

Objectives:

The overall research objective of this proposal is to fill a critical information gap regarding the epidemiology of STEC shedding in swine. Our central research goal is to provide descriptive epidemiology of STEC shedding in swine. We approached this goal with the following objectives:

1. ***Describe the epidemiology of STEC in swine.*** *The need addressed by this objective* is to generate this first longitudinal descriptive epidemiology of STEC in swine. *The approach* was a prospective descriptive cohort study of STEC shedding in finisher swine.

2. ***Characterize STEC isolates from swine.*** *The working hypothesis* was that pigs will harbor multiple different serotypes, as well as genotypes of STEC that have been associated with human disease. *The approach* was phenotypic and genotypic characterization of STEC isolates from swine.

3. ***Develop an infectious disease model of transmission of STEC from pig to pig.*** *The objective* was to develop a preliminary model of STEC transmission in finishing swine. *The approach* was development of a S-I (Susceptible-Infected) transmission model of STEC in growing swine.

Materials & Methods:

Farm Selection: One convenience sampled swine production company participated in the study. Criteria for inclusion was: 1) Evidence of the farm being STEC positive (determined by pooled fecal samples prior to study initiation) 2)All-in, all-out production 3) Farrow-to-finish production, 4) Willingness to allow access to farm sites to collect fecal samples and to share production records.

Study Design: The study design was a longitudinal descriptive cohort study. Within the production company, 3 cohorts of finisher swine (1000 head inventories in each cohort) will be selected. We will select 3 barns (1 cohort per barn).

Sampling for microbiological tests: For every cohort (n=3), 50 randomly selected pigs were individually identified using ear tags (3 cohorts X 50 pigs -150 pigs total). For each cohort, these 50 pigs were individually sampled during the finisher placement. The first sample was obtained within 2 days of pig placement in the finisher barn and then every 2 weeks after placement for a total of 8 sample periods. This duration was selected as a compromise between the logistically and financially challenging weekly sampling and the potentially too infrequent approach of monthly sampling. Experimental studies of O157:H7 STEC infection in swine had demonstrated that pigs may shed at least 2 weeks and up to 2 months post-infection (Cornick and Helgerson, 2004; Cornick and VuKhac, 2008). Fecal samples were shipped overnight on ice to the laboratory at USDA ARS ERRC.

Microbiological Methods: Enrichment of fecal samples. All fecal sample enrichment took place at the USDA ARS ERRC facility. Fecal samples were enriched based on a modification of the procedure described by Grant et al, 2009. All samples were stored at 4°C and tested within 24 h of arrival to the laboratory. Five grams of each fecal sample were placed into Bagfilter bags (Spiral Biotech, Inc., Norwood, MA) containing 95 ml of TSB pH 3 in a filter Stomacher bag. The bag was subjected to pummeling in a Stomacher for 30 sec, and then incubated at room temperature for 10-15 min. One hundred milliliters of TYTP (TSB + 12 g/liter yeast extract, 12.5 g/liter Trizma Base, and 1 g/liter 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 extraction and PCR: One milliliter of the enrichment was subjected to DNA extraction. DNA extraction was performed using the PrepSEQ Rapid Spin Sample Preparation Kit (Life Technologies Corporation, Carlsbad, CA) according to the manufacturer's instructions.

The multiplex PCR assay was performed using mISO primers and probes targeting the *stx* (detect stx_1, stx_2 and all variants except stx_{2f}) and *eae* genes and the TaqMan® Environmental Master Mix 2.0 (Life Technologies). The PCR assay was performed in an Applied Biosystems 7500 thermal cycler, and the cycling protocol consisted of 50˚C for 120 seconds, followed by 95˚C for 10 minutes, and then 40 cycles of 95˚C for 15 seconds and 60˚C for 60 sec.

Colony isolation: Enriched samples positive for *stx* were plated onto CHROMagar STEC (DRG International, Inc., Mountainside, NJ). Three presumptive positive colonies per plate were picked and confirmed as STEC using the $stx_{1/2}$/*eae* PCR assay as described above.

Characterization of isolates: One colony per pig per collection (n=194) were sent to the *E. coli* Reference Center at The Pennsylvania State University (University Park, PA) for serotyping. Although detection of other virulence genes were initially proposed, these were not completed due to the higher than expected isolation rate of STEC in this study resulting in insufficient funds to pursue further characterization. We have identified additional resources to pursue characterization of virulence genes for these STEC isolates from another source.

Statistical Analysis: Sample size: The outcome of interest was prevalence of a STEC positive pig at any one sampling period within a cohort. Based on previous data (Fratamico et al, 2004), we based our sample size on an estimate an overall prevalence of 30%. A sample size of 50 pigs allows estimation of 30% prevalence $\pm$ 10% with 90% confidence.

Descriptive data including prevalence at each collection period, incidence, duration and pattern of shedding will be tabulated and graphically represented. Descriptive data regarding the phenotypic (O serogroup) and genotypic characteristics (shiga-toxin gene subtype) of the STEC were tabulated.

The development of a transmission model (Objective 3) was conducted but altered based on data to a SIS model (SIS) using ModelMaker 4 software. Differential equations were based on that described by Vynncky and White, 2012. Assumptions regarding STEC transmission were adapted from Dorpfer et al (2012). Pigs that were detected as shedding more than 1 STEC serotype were excluded from the analysis. Recovery rate was estimated using study data based on Kaplan Meier survival estimates (STATA 12.0). The 50^{th} percentile shedding duration was 14 days.

Results:

Objective 1. Describe the epidemiology of STEC in swine.

The overall incidence of STEC in was 65.3% (98/150). The proportion of pigs detected as shedding STEC in their feces by age at collection and cohort are shown in Figure 1. The proportion of positive pigs at each collection ranged from 0-62%. STEC was detected in samples from 31 out of the 50 pigs in cohort 1, 27 out of the 50 pigs in cohort 2, and 40 out of the 50 pigs in cohort 3 (incidence ranging from 54-80%). The pattern of shedding within cohorts appears to follow a typical outbreak curve pattern. Pigs were detected positive at all age groups when all cohorts are considered.

The number of times an individual pig was positive ranged from 1-4 collection periods. Overall, most positive pigs were detected as STEC positive only once (54/98 positive pigs). There was some variability by cohort in the

distribution of the frequency of detection per pig by cohort (Figures 2, 3, and 4). Only one pig was detected positive for STEC at 4 collections.

Objective 2. Characterize STEC isolates from swine.

No STEC isolates harbored the intimin gene (*eae*). One STEC isolate per pig per collection (n=191) was submitted for determination of the stx subtype and for O serogrouping. The distribution of STEC isolates by serogoup and stx subtype are shown in Table 1. There were nine O serogroups identified, with the majority of isolates (82.2%) being O59 (n=157). The next most common serogroup were those that were not able to be identified by O group (n=28, 14.7%). Most isolates harbored the shiga-toxin gene stx_{2e} (98.5%). .

Objective 3. Develop an infectious disease model of transmission of STEC from pig to pig.

The estimated R_0 for STEC transmission was 2.54. Figure 3 shows the graphical output regarding STEC transmission based on the data from this project. After 47 days the susceptible and infected populations become stable.

Discussion:

These data represent, to the best of our knowledge, the first longitudinal study of STEC in US finishing swine. The results of this study indicate that swine shed STEC at relatively high rates that are similar to that reported in cattle. In this farm, a single O serotype predominated, but nine different O serotypes were identified, suggesting a diversity of O types can be present in the same farm. Although the data is not shown, individual pigs were also detected to be shedding more than one serotype. The most predominant O serotype in this study (O59) has been previously reported to be isolated from animal products (Werber, 2008; Hussein, 2007). Among other identified O serotypes, 6 (O20, O49, O89, O115, O119, and O167) have been identified in cases of human illness (Kappeli, 2011, WHO 1998, Werber , 2008). Serotypes O15 and O20 were previously reported in swine by Fratamico et al (2008). It is important to understand that serotype by itself is not a reliable predictor of the risk for causing human illness.

Understanding what STEC characteristics are associated with the ability to cause human illness is a challenge. There are more than 300 known serotypes of STEC, and not all are associated with human clinical illness. There is a significant challenge in differentiating non-O157 STEC isolates based on their risk for causing human illness. Although, by definition, these isolates harbor *stx*, and the *stx* encoded Shiga toxin is the main virulence trait for Hemolytic Uremic Syndrome (HUS), not all STEC have the ability to cause human clinical disease. Other virulence determinants are necessary to cause human clinical illness.

Serotype is one mechanism for determining the risk posed by an STEC strain for human clinical illness. The USDA Food Safety & Inspection Service primarily bases their risk assessment on O serotype frequency distribution in cases of human illness. (USDA, 2011) Based on CDC data, more than 80% of reported and serotyped non-O157 STEC isolated from human illnesses in 2003-2006 were represented by the O groups O26, O45, O103, O111, O121, and O145 (Gould, et al 2009, reported by USDA 2011), suggesting a linkage between O serogroup and human illness risk. Yet, more than 30 serotypes were represented in the remaining non-O157 isolates from human cases during that time period.

Other investigators have suggested seropathotypes and virulence profiles as means for molecular based risk assessment of STEC potential to cause human illness. Karmali et al (2003) developed a risk profile based on serotype, incidence of human disease, association with outbreaks and severity of clinical symptoms. Further research by Coombes et al (2008) leveraged the seropathotype classification to further evaluate the genetic basis for virulence. They evaluated the association between the presence of specific pathogenicity islands (O-Islands) and *nle* effector genes (non-LEE

encoded effectors). There was an association between specific pathogenicity island presence and HUS and outbreak origination of strains. The number of *nle* genes harbored was also associated with whether that strain originated from cases of HUS and outbreak events, with increasing number of *nle* genes associated with increased risk for an isolate originating from cases of HUS and outbreak related strains.

The recent O104:H4 outbreak in Germany associated with sprout consumption is an example of the challenges associated with understanding the risk of human illness associated with STEC (Steulins et al, 2011). This isolate, which was an Enteroaggregative *E. coli* (commonly associated with travelers' diarrhea and diarrhea in young children) that harbored stx_2, was associated with a very large outbreak of severe HUS with an unusual patient demographic risk profile (patients were more likely adult women). Further, it lacked the pathogenicity island (initimin (*eae)* and haemolysin (*hly)* negative) commonly associated with HUS illness and outbreak strains. Further research on determining the virulence gene profiles of the STEC isolates from this study is planned in order to more fully understand the potential for causing illness.

We have constructed a preliminary SIS model regarding transmission of STEC in finishing swine. This preliminary model is significantly limited by the nature of the single study provided here, but will provide a basis for integrating future data on STEC transmission in swine populations. Yet, it suggests that susceptible and infected populations stabilize approximately 47 days after initial exposures. Future work integrating more data from more groups of swine, as well as evaluation of transmission dynamics by serotype/virulence type and pursuit of stochastic models is planned.

In addition to challenges regarding determining the pathogenic potential of STEC in humans, there are limitations of this study that should be considered. The study was conducted on 3 cohorts of swine from one production company. Although the system is very similar in production practices to most conventional swine production in the United States, further investigations are needed to understand whether the results found here are applicable to other swine farms. Knowledge from this study on the incidence and duration of shedding of STEC, as well as the serogroup diversity of STEC in swine provides critical preliminary data to allow for the design and implementation of future research to understand the epidemiology of STEC in swine.

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Figures and Tables

Figure 1. Prevalence of Shiga-toxin producing *Escherichia coli* in finishing swine by age at collection and cohort.

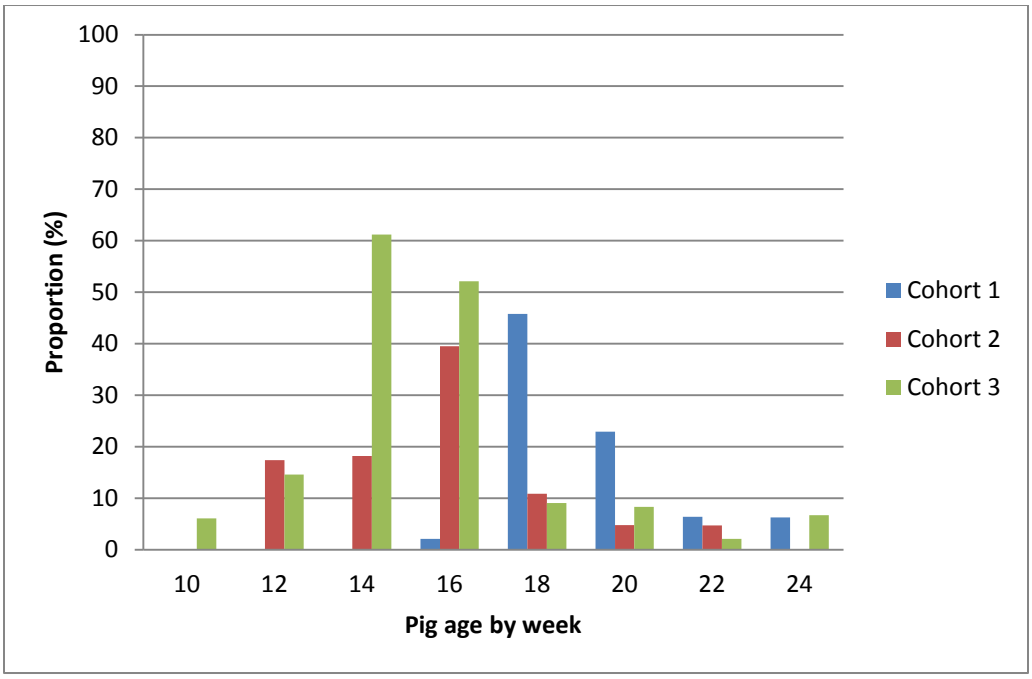
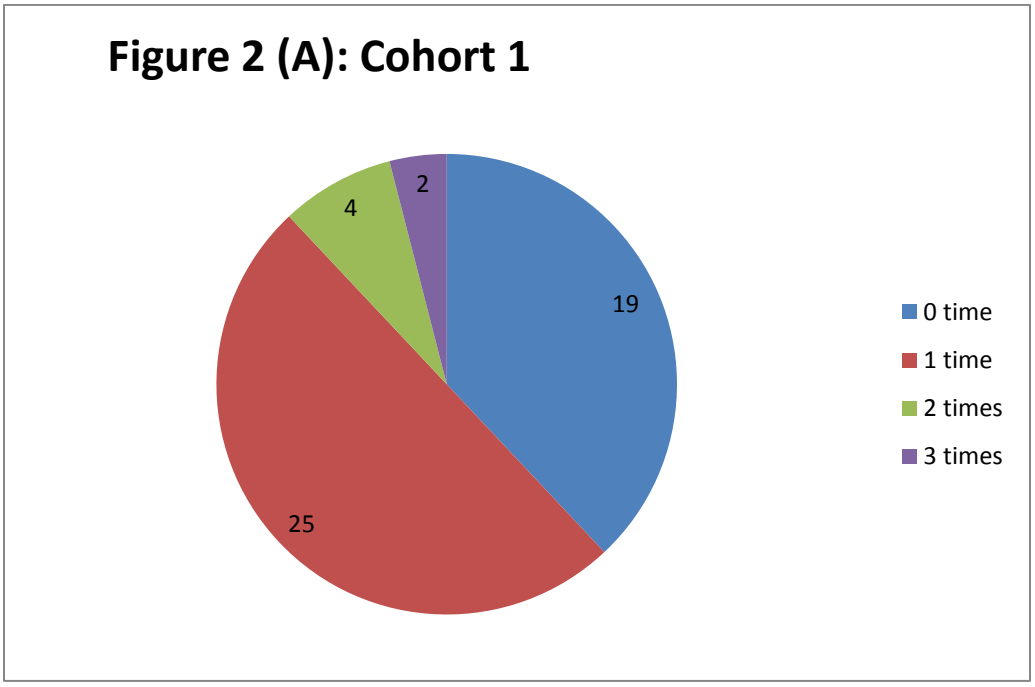


Figure 2. The number of times a pig was detected shedding Shiga-toxin producing Escherichia coli for cohorts 1(A), 2 (B) and 3(C).



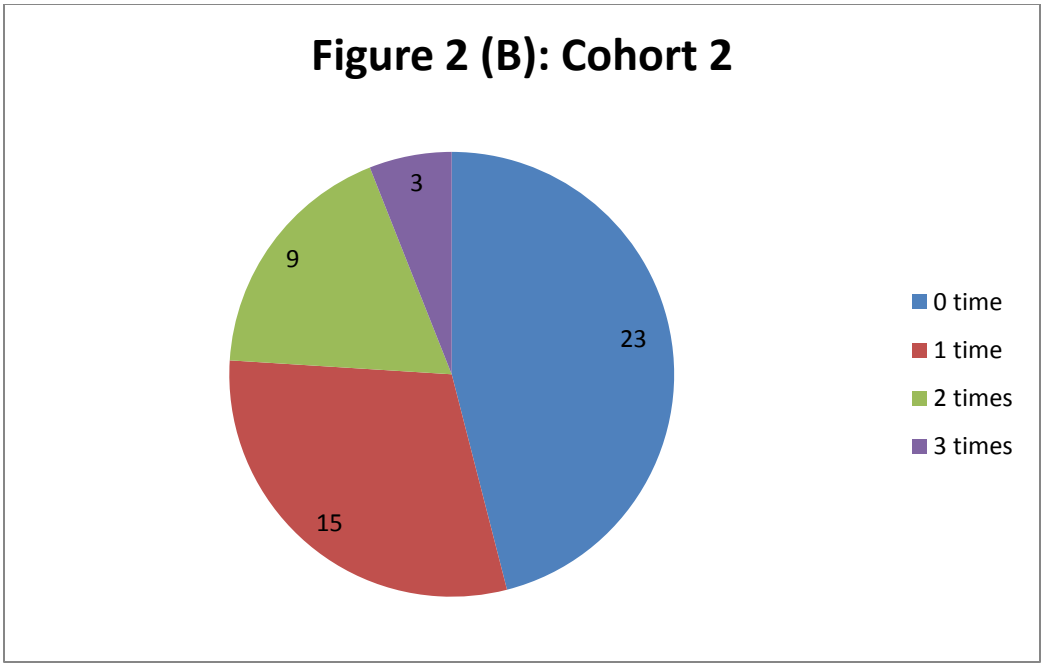


Figure 2 (B): Cohort 2
3
9
23
15
0 time
1 time
2 times
3 times

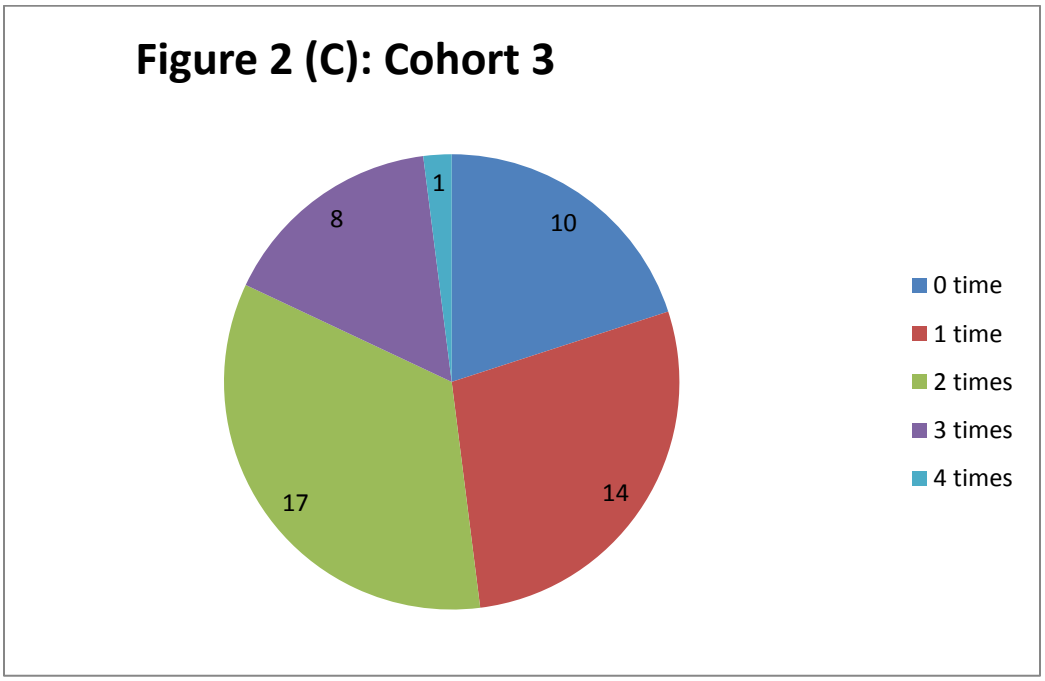


Figure 2 (C): Cohort 3
1
8
10
17
14
0 time
1 time
2 times
3 times
4 times

Table 1. Serogroup and Shiga toxin gene subtype distribution for STEC isolated (n=194) from all swine included in the study.

Serogroup	stx1	stx2 not 2e	stx2e
O15	0	0	2
O20	0	0	1
O49	0	0	1
O59	0	0	157
O89	0	0	1
O98	1	0	0
O115	0	0	1
O119	0	0	1
O167	0	0	1
negative	0	2	26
Total	1	2	191

Figure 3. Plot of STEC new infections, susceptible and infected populations based on preliminary data from this project.