ROLE OF MUSCOID FLIES IN THE ECOLOGY OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) IN CONFINED CATTLE ENVIRONMENTS

by

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M.Sc., Tribhuvan University, 2012

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Entomology
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KANSAS STATE UNIVERSITY
Manhattan, Kansas

2015

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Abstract

House flies (*Musca domestica* L.) and stable flies (*Stomoxys calcitrans* L.) are insects of medical and veterinary importance. House flies are recognized as mechanical vectors of human foodborne pathogens and stable files are known for their painful bites resulting in reduction of body weight gain and milk production in cattle. The larval development of both fly species takes place in decaying organic materials (primarily animal manure), resulting in large fly populations in confined cattle environments. Shiga toxin-producing *Escherichia coli* (STEC) are a major foodborne pathogen. Cattle are the asymptomatic reservoir of STEC with bacteria being released to the environment via their feces. STEC O157 is the main serogroup causing human illness. However, infections with non-O157 STEC are increasing: more than 70% of non-O157 infections are caused by six serogroups of non-O157, referred as "Big six" (O26, O45, O103, O111, O121, and O145). In addition, there was a large 2011 outbreak in Europe caused by STEC O104. The objectives of my thesis were: 1) To assess the prevalence of seven serogroups of non-O157 STEC (O26, O45, O103, O104, O111, O121, and O145) (STEC-7) in house flies and stable flies collected from confined cattle environments; 2) To investigate the vector competence of house flies for non-O157 STEC-7. A total of 463 house flies from feedlots and dairies from six states, and 180 stable flies collected from a feedlot in Nebraska were processed for the isolation and identification of STEC-7 using a culture-based approach followed by PCR for the confirmation of serogroups, and virulence genes. A total of 34.3% of house flies and 1.1% of stable flies tested positive for at least one serogroup of *E. coli* of interest, and 1.5% of house flies harbored STEC with the Shiga-toxin gene (*stx1*). No STEC were detected in stable flies. Vector competence bioassays for non-O157 STEC revealed that house flies can carry non-O157 STEC for at least six days with the exception STEC O145. Overall, the findings of this research

demonstrate that house flies, but not stable flies, likely play an important role in the ecology and transmission of non-O157 STEC in confined cattle environments.

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Acknowledgements

This research was supported by the U. S. Department of Agriculture National Institute for Food and Agriculture Grant No. 2012-68003-30155. I express sincere thanks to my major adviser Dr. Ludek Zurek for his continuous support and encouragement throughout my study. I thank my committee members Dr. Anuradha Ghosh and T. G. Nagaraja for helpful discussions, guidance, and input into my research. I also would like to acknowledge Dr. Natalia Cernicchiaro for the collaboration and Ms. Jessica Thompson, Ms. Megan Spencer, Drs. Roger Moon, Justin Talley, Phillip Kaufman, Alec Gerry, and Samuel Ives for providing the flies for my experiments. I also want to acknowledge Ms. Jessica Thomson for her help in the laboratory. Finally, I am forever grateful to my husband Dipak Giri who always gave me the encouragement and support I needed.

Chapter 1 - Literature Review

Biology, behavior and management of house flies and stable flies

The house fly (*Musca domestica* L.) is a synanthropic, non-biting insect of order Diptera and family Muscidae (Moon 2002). House flies are medium sized (5-12 mm long) insects with gray to black color and have been observed in close association with humans and livestock all around the world except Antarctica (Moon 2002). It has complete metamorphosis and passes through four developmental stages: egg, larva, pupa, and adult.

Adult female deposits 50-150 eggs at a time in a cluster on suitable breeding sites at an interval of 2-4 days. A female lays as many as 500 eggs during her lifetime (Williams 2009). Larval development habitats include- human garbage dumps, open privies, livestock manure, soiled bedding, poultry litter, and waste around fruit and vegetables processing plants (Moon 2002). The microbial community, which is essential for the larval development is present in these materials (Zurek et al. 2000). The eggs are small (2-3 mm) and white in color, hatch into the larva within 1-2 days and undergo three instars of larval stages. Egg mortality increases greatly when humidity of the substrate decreases below 90% (Williams et al. 1985). The larval development of house flies takes about 7 days in warmer weather (Williams 2009). The optimum temperature for larval development is 35ºC (Williams et al. 1985). The larva changes into non-feeding immobile pupa stage and adult flies emerge from the pupa within 3 to 10 days depending on the temperature (Williams et al. 1985). Adult house flies begin feeding within 24 hours (h) and are capable of mating soon after emergence (Williams 2009). It takes about 7 to 14 days to complete the lifecycle. While two or more generations are possible in warm months, 10-12 generations per year occur in the temperate climates (Williams 2009). Although, house flies were

reported to fly up to 20 miles, they, mostly (90% of time) remains within the range of 2 miles (Levine and Levine 1991).

House flies are one of the important nuisance pest and acts as a mechanical vector of several pathogens of human importance (Graczyk et al. 2001). They harbor pathogens externally (on the body surface) as well as internally (in the gut). The population dynamics of house flies varies with the seasons showing peak in the summer and early fall in livestock environments (Lysyk 1993, Mullens and Meyer 1987, Talley et al. 2002). House flies can degrade the appearance of facilities and animal products from their unsightly regurgitation and fecal spots (Williams 2009). House flies can be a source of nuisance to the surrounding residential places if animal facilities have large population due to lack of management. Although house flies become quite abundant where livestock, poultry and companion animals are housed, their direct effect on animal health is comparatively less important (Moon 2002).

Management: Sanitation and hygiene are the most important practices to manage flies in and around livestock facilities. Manure should be removed, or spread thin to dry up, cleaned around the feed bunks, under fence and gates, water systems, and at the end of mounds. Removal of larval developmental habitat reduces the fly population greatly. The second control measure includes insecticides; however, development of resistance to insecticides makes it less effective (Kaufman et al. 2001, Marcon et al. 2003). The phagostimulant and visual lure (dark blue) designed to create toxic baits were shown to work effectively in controlling indoor house flies (Khan et al. 2013). Biological control mostly includes parasitic wasps. Climate and geographic location affects the efficiency of some parasitic wasp in reducing the fly population. Wasp of family Pteromalidae and genera *Muscidifurax* and *Spalangia* were found to be effective

(Williams 2009). Proper design and management of livestock farms are essential to reduce the effect of flies to cattle.

Stable flies: The stable fly (*Stomoxys calcitrans* L.) is another insect in Muscidae family. They are worldwide in distribution and are more common in temperate regions but are found in lower number in cold climates. They are blood feeders and both sexes suck blood which brings them in close relationship with animals (Foil and Hogsette 1994). The stable fly is noted for its painful bite because of its mouthparts (piercing and sucking). They are also called as biting house flies because of their resemblance to house flies (Moon 2002). In the U.S. stable flies have got some regional names other than stable flies such as beach flies (due to their presence in beaches), dog flies (as they bite in dog's ears), and lawn mower flies (as the larva develops in the grass mats under lawnmowers) (Broce 2006). The primary host of this fly is cattle during most of the year, however, humans became primary host on beaches during the late summer and early fall making outdoor recreation unpleasant (Hogsette et al. 1987). Flies usually feed below the knee, and also move to the sides, and back if present in large number (Hogsette et al. 1987).

Like house flies, stable flies are also holometabolous insect with four stages in their life cycle: eggs, larvae, pupae, and adults but are slightly slower in development than house flies because of their larval habitat in aged manure (Broce and Haas 1999). The female lays up to 800 eggs in her lifetime, 60-130 eggs in clutches at a time. The stable fly larva develops in variety of media including cattle manure, spilled feed, silage, composting grass clippings, and in mixture of hay and manure. Blood meal is essential for the proper survival and reproduction of the stable fly (Jones et al. 1992). This fly on an average ingests 11-15 µl of blood/meal and female sucks little higher amount than male (Schowalter and Klowden 1979). Eggs hatch in 12-24 h after oviposition, and first instar larva forms, which molts and grows in to second and third instar

larva in 12-13 days at the temperature of 27ºC. Pupa changes into adult in approximately 7-10 days (Foil and Hogsette 1994). The complete life cycle takes about three weeks in summer days under optimum conditions of nutrient, humidity, and temperature. Mating may occur as early as 2 days after emergence. Adult flies are gray in color with four longitudinal black stripes on the thorax and checkerboard marking in abdomen in the ventral surface (Williams 2009, Foil and Hogsette 1994). They are smaller than house flies (5-7 mm long) and are sometime mistaken with horse flies because of their biting nature (Foil and Hogsette 1994).

Painful bites of stable flies make animals restless and make them to defend by moving their body parts mainly head, ears, tail, and legs. Animal tend to stay closer to each other, inside water and hiding in the forest to avoid biting (Baldacchino et al. 2013). The annoyance of stable flies mainly cause loss of energy, reduction in feeding time and feed intake, and stress. There is high economic impact of stable flies in animal industry due to their painful bites that cause reduction in milk production in dairy cattle and affects weight gain in beef cattle reducing their feed efficiency. A loss of about $2.2 billion every year has been estimated in the U.S. due to biting of stable flies to dairy cattle, pasture cattle, cow-calf herds, and feedlot cattle (Taylor et al. 2012). The population density of both house flies and stable flies tend to increase during the spring season, stabilize in the summer and early fall, and decrease during fall to spring (LaBrecque et al. 1972).

Management: Controlling stable flies is difficult. Sanitation or the removal of larval developmental sites is the primary mean to control stable fly population around confined animals. All the decaying vegetable matter should be burned, buried, or covered with some agents so that it would not be accessible to stable flies for oviposition and larval development (Shipley 1915, Broce 2006). Minimization of residue accumulation by moving the wagon a short

distance every two week and routine removal of rolled hay residues eliminate or reduce the population of stable flies greatly along with stacking and burning of active rolled hay (Foil and Hogsette 1994).

Other controlling measures include use of parasitoids. Parasitoid wasps can be used to reduce stable fly populations in the field (Broce 2006). Chemical control of stable flies is not very effective as they develop resistance to some of the insecticides. They are shown to be resistant to insecticide organophosphate (dichlorovos and stirofos) and pyrethroid (permethrin) in Kansas (Cilek and Greene 1994). The frequency of resistance observed was 2 to 100%. Using insecticide to animal leg is just a relief for few days as it is removed easily from animal body by vegetation and water (Campbell and Hermenussen 1971). Insecticide applicators such as oilers, dust bags, ear tags are also not effective for stable flies (Broce et al. 2005).

Traps are useful to control the indoor fly population and represent a common method to monitor the outdoor fly population. A comparative study to determine the efficiency of two traps showed BiteFree prototype (polyethylene terephthalate) trap to be more efficient than the Alsynite trap of Broce and Olson (Taylor and Berkebile 2006). Different fabric targets treated with λ-cyhalothrin or zeta-cypermethrin were found to be effective in controlling stable fly populations (Hogsette et al. 2008). However, lab colony flies were tested for the efficiency and no information is available about the usefulness of this trap in the field.

Role of house flies and stable flies in pathogen transmission

House flies (*Musca domestica*) are synanthropic insects of medical and veterinary importance. They are more than nuisance because of their ability to carry and transfer pathogens. Transmission of a pathogen takes place mainly due to their unique mode of feeding that includes regurgitation. In addition, fecal deposition and attachment in the outer body parts also aid

pathogen transmission (Graczyk et al. 2001). House flies are recognized mechanical vector of human and animal pathogens that include bacteria, virus, and protozoans (Graczyk et al. 2001, Fetene and Worku 2009, Förster et al. 2009). House flies feed on liquid food through their sponging mouth parts but when they feed on solid food, food needs to be liquefied by regurgitating its gut content which includes microbes.

Bacteria that has been isolated from house flies include *Salmonella* spp. (Bidawid et al. 1978, Olsen and Hammack 2000, Mian et al. 2002, Ugbogu et al. 2006), *Shigella* spp. (Bidawid et al. 1978, Levine and Levine 1991, Ugbogu et al. 2006), *Klebsiella* spp. (Fotedar et al. 1992, Sulaiman et al. 2000), *E. coli* O157 (Moriya et al. 1999, Iwasa et al. 1999, Alam and Zurek 2004), *Vibrio cholerae* (Fotedar 2001), *Campylobacter fetus* (Rosef and Kapperud 1983), *Aeromonas caviae* (Nayduch et al. 2001), enterococci (Macovei and Zurek 2006, Graham et al. 2009), and *Listeria* spp. (Pava-Ripoll et al. 2012). House flies are able to transfer pathogens from the feces of animal and human origin (Fotedar 2001, Ahmad et al. 2007, Nayduch et al. 2001). House flies collected from garbage dumps in Ethiopia were found to carry helminth and protozoan parasites. Both helminths (*Ascaris lumbricoides*, *Trichuris trichiura*, hookworms, *Taenia* sp. and larvae of *Strongyloides stercoralis*) and protozoan species (*Entamoeba histolytica*/*dispar*, *Entamoeba coli*, *Giardia lamblia*, and *Cryptosporidium* sp.) were isolated from the body surface and the intestinal contents of flies (Getachew et al. 2007). Pathogens *Ascaris lumbricoides, Entamoeba coli*, *Giardia lamblia* were also recovered from external body surface of house flies collected from restaurants, butcheries, and supermarkets in Iran and Nigeria (Motazedian et al. 2014, Balla et al. 2014).

The load of bacteria in the gut of house fly is higher than on the external surface even though external body surface provides the important habitat for microbes (Barro et al. 2006).

Shane et al. (1985) recovered only 20% of bacteria from feet and ventral surface; however, 70% were from viscera. Similarly, more parasites were isolated from the gut of flies than the external surface (Getachew et al. 2007, Förster et al. 2009).

House flies come in direct contact with pathogens because of their developmental habitat. Microbial community is essential for their larval development (Zurek et al. 2000). One hundred and two species of bacteria isolated from the gut of 65 house flies collected from different environments (slaughter house, garden, garbage area, public toilet, hospital, restaurant, human habitation) were shown to carry bacteria of human importance using culture dependent and culture independent methods (Gupta et al. 2012). *Klebsiella*, *Aeromonas*, *Shigella*, *Morganella*, *Providencia*, and *Staphylococcus* were identified as the most abundant bacterial genera of human importance. The ability of house flies to transmit pathogens has been observed in many studies. Inoculated house flies, when released into the poultry room, mechanical transmission of *Salmonella enterica* serovar Enteritidis to chickens and vice versa were observed by Holt et al. (2007). The contamination of house flies with *Salmonella* takes place within 24 to 48 h post challenge of hens and bacteria persist for two weeks in the house fly body (Holt et al. 2007). Fate of *Salmonella* in adult house flies depends on the number of factors such as size of inoculation, and the successful establishment takes place up to 10^4 bacterial cells of *Salmonella*. Presence of other bacteria may lead to the elimination of *Salmonella* from the gut of house flies (Greenberg et al. 1970).

Indirect correlation of human infections and disease (diarrhea and trachoma) with muscoid flies was studied by Emerson et al. (1999). The disease prevalence was studied before and after the control of flies using deltamethrin for three months in two pairs of villages. The strong positive correlation was observed with populations of flies and incidences of trachoma

and diarrhea. There were 22% fewer diarrheal cases with 75% reduction in the fly populations. Similarly, 95% reduction in the house fly population led to 23% reduction in diarrheal infections in Pakistan and Israel (Chavasse et al. 1999, Cohen et al. 1991).

In the laboratory, house flies can carry pathogen *Yersinia pseudotuberculosis* up to 36 h (Zurek et al. 2001), *Aeromonas caviae* up to day 8 (Nayduch et al. 2002), and *Staphylococcus aureus* up to 6 h in their gut post infection (Nayduch et al. 2013). In addition, Kobayashi et al. (1999) and Doud and Zurek (2012) showed that house flies act not only as simple mechanical vectors but also help to carry out multiplication of bacteria such as *E. coli* O157 in the labellum (Koyabashi et al. 1999), and GFP expressing *Enterococcus faecalis* in the crop (Doud and Zurek, 2012). *Enterococcus faecalis* can multiply in the crop of house flies and can persist up to 96 h post infection.

Adult house flies collected from fast food restaurants were shown to carry antibiotic resistant enterococci (Macovei and Zurek 2006) and 97% of house flies were positive for enterococci with the mean concentration of 3.1×10^3 CFU/fly. Three ready to eat food items sampled from the restaurants in summer and winter were also shown to have enterococci with higher concentrations in the summer (Macovei and Zurek 2007). This coincides with the peak season of house flies. Adult house flies collected from cattle feedlots were able to contaminate ready to eat food (beef patty) in the laboratory assay (Macovei et al. 2008) indicating the role of this synanthropic fly in dissemination of bacteria. House flies were also able to transfer cysts of *Toxoplasma gondii* that cause toxoplasmosis under laboratory conditions (Wallace 1971). Zurek et al. (2001) studied the vector competence of the house fly for *Yersinia pseudotuberculosis* and found that the house flies were able to carry this pathogen in their gut for 36 h of post

inoculation. In addition, flies could transfer *Yersinia* to sterile environment (sterile TSB) until 30 h of post inoculation indicating the potential of flies as a mechanical vector of this pathogen.

Twelve species of flies including house flies from animal environments were shown to carry enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC) in their external surface (Förster et al. 2007) and were able to transfer these bacteria to agar plates. Bacteria were isolated from the proboscis, legs, and feces (Barro et al. 2006).

The house fly can also act as a vector of *Chlamydia trachomatis*, causative agent of trachoma. This pathogen was detected up to 2 h from leg and/or proboscis and up to 6 h in the intestine of flies in the laboratory conditions (Forsey and Darougar 1981). The possibility of house flies to be a vector of *Helocobacter pylori* was studied by Grübel et al. (1997). Bacteria were detected in the gut of flies up to 30 h post inoculation. Antibiotic resistant *Klebsiella* spp. was detected in the house flies from hospital (Fotedar et al. 1992). Similarly, *Vibrio cholerae* was also isolated from house flies in India and this strain was similar to those isolated from the human feces (Fotedar 2001). Development of house flies was supported by enterococci; however, some species (*E. faecalis* and *E. faecium*) did not support the growth of larva and bacteria were detected in teneral adults indicating the transstadial transmission (Ghosh et al. 2014).

Stable flies are one of the important pestiferous insects because of their painful bites and nuisance to the animals in confinements and pasture land (Moon 2002, Taylor et al. 2012). Reduction in the feed efficacy, weight gain, and milk production in animals are the major issues among cattle in United States due to stable fly bites (Taylor et al. 2012).

There have been several laboratory studies investigating the stable fly role in transmission of pathogens. Equine infectious anemia, caused by a retrovirus responsible for

infection in horses (characterized by fever, anemia, edema, thrombocytopenia) were shown to be mechanically transmitted by this fly (Foil et al. 1983, Hawkins et al. 1973). Stable flies inoculated with African swine fever virus were able to transmit this pathogen to susceptible pigs for 24 h and this virus survived in the stable fly for 2 days (Mellor et al. 1986). Thus, stable fly may help in the dissemination of this virus in swine environments. West Nile fever virus (WNV) was detected in stable flies when allowed to feed upon white pelican (Johnson et al. 2010). Doyle et al. (2011) studied the potential of this fly to transmit the WNV. Results suggested that stable flies can mechanically transmit this virus for 6 h but virus cannot undergo multiplication in the stable fly and may act as short time mechanical vector. Stable flies also mechanically transfer Rift valley fever virus (Hoch et al. 1985, Turell et al. 2010) and bovine diarrhea virus to hamsters and cattle (Tarry et al. 1991). All these studies were conducted under the laboratory conditions and reports of natural transmission are not documented.

Stable flies are reported to be the potential vector of bacteria as well. They are shown to be a vector of *Bacillus anthracis*, causative agent of anthrax and were able to transmit bacteria from dead or sick animals to healthy ones (Hugh-Jones and Blackburn 2009). *Cronobacter* (*Enterobacter*) *sakazakii* were detected in stable flies (0.2% prevalence) and were able to carry it at least 20 days post inoculation, and contaminated their food source with this pathogens under laboratory condition (Mramba et al. 2006, Mramba et al. 2007). In addition, the development of stable flies is supported by this bacterium in manure. Inoculated stable flies were able to transmit *Dermatophillus congolensis* to healthy rabbits (Richard and Pier 1966). Thirty-three distinct bacterial species including *E. coli*, *Staphylococcus aureus* were identified from stable flies in Brazil from the dairy cattle environment (Castro et al. 2007) in three distinct parts: cuticle, mouth parts, and the digestive tract. The same author also isolated Shiga toxin-producing

Escherichia coli (STEC) from flies in dairy farms in Brazil (discussed later in this chapter). Stable flies were shown to transmit other bacteria as well, *Anaplasma marginale, Coxiella burnetii, Besnoitia besnoiti, Leishmania tropica,* and helminths such as *Habronema microstoma* in the laboratory conditions (Baldacchino et al. 2013). Isolation of clinically important microbes made stable fly potentially important insect of medical and veterinary importance. However, further research is essential to explore if stable flies pose threat for the transmission of bacteria and viruses in the field (natural conditions).

Shiga-toxigenic *Escherichia coli* (STEC) O157 & non-O157 (pathogenicity & epidemiology)

Escherichia coli are rod shaped, Gram negative, facultative anaerobic commensal bacteria in the *Enterobacteriacae* family that commonly reside in the intestine of mammals and other animals. Pathogenic *E. coli* strains are classified mainly to six types based on their pathogenic feature. They are Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC) Entropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Diffusely adherent *E. coli* (DAEC), and Enterohaemorrhagic *E. coli* (EHEC) (Croxen et al. 2013). Serotyping of *E. coli* is based on the Kauffman classification scheme which includes O (somatic/Lipopolysaccharide), H (flagellar) and K (Capsular) antigen profiles (Croxen et al. 2013). *Escherichia coli* are classified based on the O:H antigen combination, serotypes.

Shiga toxin-producing *Escherichia coli* (STEC) are the subset of EHEC also are referred as Verotoxigenic *E. coli* (VTEC) and are important foodborne pathogens worldwide. They are responsible for illnesses such as diarrhea, bloody diarrhea, hemorrhagic colitis (HC), thrombotic thrombocytopenic purpura (TTP) and life-threatening hemolytic uremic syndrome (HUS), (Tarr et al. 2005). The STEC refers to the *E. coli* that harbor the *stx1* or/and *stx2* gene whereas EHEC

refers to *E. coli* having the Shiga-toxin gene along with *eae* which encodes intimin, an essential protein for attachment to the epithelial wall (Melton-Celsa et al. 2012). One serotype of STEC, O157:H7 was identified in 1982 from the contaminated beef patties (Riely et al. 1983). Many outbreaks and sporadic cases of STEC occurred in the U.S. as well as worldwide after the discovery of this bacterium infecting thousands of people. In the United States, STEC O157 was recognized as adulterant in 1994, and more recently the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) declared six non-O157 STEC serogroups (O26, O45, O103, O111, O121, O145) (STEC-6) as adulterants in raw, non-intact beef products (Pihkala et al. 2012). Although O157 is the major serogroup of STEC for human infection, more than 200 serogroups of non-O157 STEC are reported to cause STEC illness (Caprioli et al. 2005, Hedican et al. 2009, Käppeli et al. 2011). Both O157 and non-O157 are associated with multiple outbreaks infecting thousands of people every year globally, mainly affecting children of 0-4 years old, immunocompromised adults, and elderly people (Majowicz et al. 2014).

Shiga-toxigenic *E. coli* are estimated to cause more than 265,000 illnesses each year in the United States alone with 30 deaths (Scallan et al. 2011). Human infections with the non O157 STEC are increasing in recent years (Brooks et al. 2005, Hughes et al. 2006, Gould et al. 2013, Johnson et al. 2006). Six serogroups of non O157 STEC (O26, O45, O103, O111, O121, and O145) are responsible for more than 70% of total non-O157 infections to humans (Gloud et al. 2009, CDC 2011) and are called "Big Six". In addition, one rare serogroup of non-O157 STEC, O104 was responsible for the large outbreak in Europe in 2011 resulting in death of 39 people (Frank et al. 2011). Non-O157 infections were reported to be milder than O157 (Hedican et al. 2009). However, other studies showed equal severity of O157 and non-O157 infections (Hermos et al. 2011).

An undercooked ground beef is the main source of human infections and fecal contamination of carcass in slaughter is the main route for *E. coli* to enter the food chain and cause infection to people (Rangel et al. 2005). Outbreaks involving ground beef are peak in summer and are associated most frequently in community level (48%) followed by picnics and camps (11%), individual residences (9%), and schools (5%) (Rangel et al. 2005). Human STEC infection is typically through fecal oral contamination. People get sick after ingesting bacteria by a direct or indirect contamination of human and animal feces (Evans and Evans 1996). Severity of infections depends upon the amount of bacteria ingested and immune status of the infected person. Immunocompromised adults, elderly adults, in addition to children under the age of four were found to be affected the most (Gould et al. 2013).

The virulence factor of STEC are Shiga-toxin with two major gene families *stx1* and *stx2* along with intimin protein which is essential for the attachment to the intestine epithelium and is encoded by intimin (*eae*) (Gyles 2007) and enterohemolysin (*ehxA*) genes. The Shiga-toxin gene *stx1* shares 50-60% of genetic and amino acid homology with *stx2* (Weinstein et al. 1988) and antibodies against *stx1* do not neutralize *stx2* and vice versa (Mohawk and O'Brien 2011). They both have same mode of action but the cytotoxic dose (LD50) of these toxins are different from each other, and *stx2* appears to be more toxic and associated with HUS to humans than *stx1* (Hedican et al. 2009, Scotland et al. 1988); although, this is not always the case.

The incubation period (time from ingestion of the bacteria to development of symptoms) of STEC is about 3-4 days and in case of HUS diarrhea may turn into bloody in 1-2 days and follows by HC in 5-7 days (Melton-Caster et al. 2012). The symptoms of HUS develop in 0-15% of *E. coli* infections (Melton-Caster et al. 2012).

The pathogenesis of STEC mediated infections to humans is not well understood. The infection of STEC to humans is known to involve number of steps: ingestion, colonization in the gut, and release of the toxin and finally development of complications due to toxins, which may be fatal. People are infected after eating contaminated food or water and person to person dissemination is also possible in families and daycare centers. The infection dose of these bacteria is very low, less than 100 bacterial cells are sufficient to establish infection (Tilden et al. 1996, Kaper et al. 2004); however, it depends on additional factors such as particular STEC strain, host susceptibility and the condition of pathogen in matrix consumed (Teunis et al. 2008). Both Shiga-toxin genes the *stx1* and *stx2* consist of two subunits (AB5) toxin, subunit A is involved in cytotoxic activity and the subunit B has binding function. As they colonize, five identical B subunits of the *stx* bind to the GB3 (globotriaosylceramide) receptor of the target cells in a host, and A subunit is released to the cytoplasm through receptor mediated endocytosis and cleaves the ribosomal RNA, ceasing the protein synthesis (Melton-Celsa et al. 2012). Shiga-toxins have greater degree of toxicity in some cells and tissues (epithelial and endothelial cells) and this may be due to the availability of GB3 receptors. Local damage in the colon by *stx* cause infections in intestinal epithelium and leads to diarrhea, bloody diarrhea within 3 days of ingestion and may develop HC, necrosis, and intestinal perforation in another 1-2 days (Kaper et al. 2004). The *stx* is then carried by the blood throughout the body and binds to places where it can find the GB3 receptor such as in kidneys, which leads to HUS causing hemolytic anemia, and renal failure (Kapar et al. 2004). The toxin then inhibits protein synthesis from the endoplasmic reticulum causing cell death (Paton and Paton 1998).

Treatment of STEC with antibiotic is controversial (Carter et al. 1987, Dundas et al. 2001) and mainly includes supportive (fluid) therapy. The use of antibiotics may promote toxin

expression from the lysogenized phage that carries the *stx* gene. Moreover, the promotion of antimotility agents is not recommended as they can promote the sustained presence, and consequent toxin expression (Mohawk and O'Brien 2011).

STEC have also been detected from other animals including goat, sheep, poultry, swine, cats, dogs, and wild animals such as deer, elk, coyotes, feral swine, birds, opossums, raccoons, and bison (Shere et al. 1998, Zschöck et al. 2000, Bentancor et al. 2007, Bettelheim 2007, Jay et al. 2007, Laidler et al. 2013, Callaway et al. 2013, Persad and LeJeune 2014). Direct contact with domestic animals during handling in confined environments (cattle, swine, sheep etc.) and in other events such as rodeos, petting zoos, and agriculture fairs leads to the risk of transferring pathogens if proper hygiene is not maintained. Wild animals on the other hands are also playing role in disease dissemination. There was an *E. coli* O157 outbreak in California from feral swine feces contaminated baby spinach causing 183 illnesses among which 95(52%) people were hospitalized and 29(16%) had HUS with one death (CDC 2006, Jay et al. 2007). Recently, fresh strawberries contaminated with deer feces led to the O157 infection causing death of 2 people with HUS and 15 cases of illnesses and six hospitalizations in Oregon (Laidler et al. 2013). In addition, insects are also involved in the dissemination of STEC in the environment from different sources (Persad and Lejeune 2014) and this will be discussed later in this chapter.

Study of STEC (O157& non-O157) in confined cattle environments

Cattle are the most important asymptomatic reservoir of STEC O157:H7 in the United States. *Escherichia coli* have ability to colonize the cattle of all age (Baehler and Moxley 2000). The primary site of *E. coli* colonization in cattle is the recto-anal junction (Naylor et al. 2003, Lim et al. 2007). Cattle remain healthy despite of having STEC because of lack of vascular receptors (Gb3) for Shiga-toxins (Pruimboom-Brees et al. 2000). Colonization in the hindgut

requires the similar attaching and effacing lesions as in the humans (Baehler and Moxley 2000) and after colonization; cattle shed *E. coli* in feces intermittently (Sargeant et al. 2007). Even though cattle do not have vascular receptor for *stx*, colonization of STEC, may produce enterocyte loss, inflammation and immunosuppressive effect (cell mediated immune responses) which interfere with the host to clear the infection (Moxley and Smith 2010). Bacteria are shed for a short time (days) in case of STEC do not colonize the gut. Cattle can shed bacteria for about a month possibly due to the reinfection, close contact with other cattle, and contaminated food and water (Besser et al. 1997, Khaitsa et al. 2003). The frequency of shedding and concentration of *E. coli* O157 in feces varies greatly with different animals and animal sheds different concentrations of bacteria at different times (Robinson et al. 2009). The concentration of *E. coli* O157 was reported to be ranging from 10 to 10^9 CFU/g of feces (Munns et al. 2015). Cattle shedding *E. coli* O157:H7 in the concentration $>10^4$ CFU/g of feces are called "super shedder" (Munns et al. 2015). High concentration of *E. coli* in the feces and on the hide of animals during transportation, lairage and slaughter possess high risk of carcass contamination (Arthur et al. 2010).

The STEC O157:H7 infection to humans was started with the contaminated ground beef in 1982 (Riley et al. 1983) after that it is the main food vehicle of STEC and peaks in summer months during times of high shedding of *E. coli* O157 by the beef cattle. This is responsible for 41% of STEC associated foodborne outbreaks (Rangel et al. 2005). The prevalence of STEC has been of great focus of research in dairy and beef cattle from a long time. There has been an extensive research on STEC O157 in beef cattle because of their direct association with public health (Elder et al. 2000, Keen and Elder 2002, Williams et al. 2010) and most of the outbreaks of O157 are associated with ground beef mainly hamburgers and meat sauce (Rangel et al.

2005). However, STEC was also reported to be shaded by dairy cattle. The prevalence of STEC was reported (0.2 to 48.8%) for O157 and (0.4 to 74%) for non-O157 in dairy cattle worldwide (Hussein and Sakuma 2005). Similarly, the prevalence of O157 was detected in the range of 0.3 to 19.7% in feedlots and 0.7 to 27.3% in pastures and non-O157 in the range of 4.6 to 55.9% and 4.7 to 44.8% in feedlots and pastures respectively (Hussein 2006). There are few studies comparing the prevalence of STEC O157 in feces between beef and dairy cattle (Hancock et al. 1994, Sasaki et al. 2013) and results showed higher STEC prevalence in beef than in dairy cattle. However, Cobbold et al. (2004) reported the opposite results. Season, age, and diet may affect the shedding of non-O157 STEC. Even though most of outbreaks are associated with hamburgers and meat sauces, several outbreaks (4%) of STEC O157 and non-O157 associated with raw milk and cheese made by using raw milk were also reported (Rangel et al. 2005, Hussein and Sakuma 2005). Contamination of cattle udder and tits with feces may cause transfer of pathogens to milking machine and finally to milk and milk products (Hussein and Sakuma 2005). Pasteurization is the best method to decontaminate the milk and thus preventing STEC outbreaks (Farrokh et al. 2013). A total of 193 and 373 STEC serotypes were isolated from the dairy cattle and beef cattle respectively and 24 isolates from dairy and 65 from beef cattle origin were similar to the human isolates capable to cause HUS to humans (Hussein and Sakuma 2005, Hussein 2006).

Even though non-O157 STEC has been detected in carcasses and ground beef, not much is known about their biology, prevalence, and distribution in cattle. Non-O157 was detected in cattle feces from many cattle farms (Hornitzky et al. 2002, Renter et al. 2007, Musa et al. 2013, Cernicchiaro et al. 2013, Bibbal et al. 2015). The seasonal prevalence of non-O157 in cattle is not well understood (Barkocy-Gallagher et al. 2003). The *E. coli* O157 serogroup in feces of

cattle showed strong seasonal variation and is high during summer (Barkocy-Gallagher et al. 2003, Edrington et al. 2006, Ferens and Hovde 2011). However, no data are available yet for the concentration of non-O157 in cattle because of the lack of established quantitative protocols. In a recent study, the prevalence of non-O157 STEC was rare in summer and was not detected in winter (Dewsbury et al. 2015).

Management strategies: Pre-harvest intervention strategies to reduce the bacterial contamination in beef especially for non-O157 are limited. The best way to prevent the carcass contamination is to reduce the load of pathogens in cattle entering slaughter, which can be achieved to some extent by: clean and dry bedding, sanitation practices on farms and feedlots and housing and transportation before slaughter (Callaway et al. 2013). *Escherichia coli* are reported to persist in manure for a long time (Callaway et al. 2013), therefore manure management is critical in confined cattle environments. Areas with high cattle density were shown to associate with high non-O157 STEC incidence in Germany (Frank et al. 2008). Stress in cattle during transportation and in lairage also may play a role to increase the STEC contamination of carcasses (Callaway et al. 2013).

Other pre-harvest technique such as direct fed microbial supplements, for example *Lactobacillus acidophilus* NP51 strain were found to reduce the STEC O157 shedding significantly (Brashears et al. 2003, Loneragan and Brashears 2005). However, there was no effect of direct fed microbial (*Lactobacillus acidophilus* NP51, and *Propionibacterium freudenreichii* NP24) to the shedding of non-O157 (Cernicchiaro et al. 2014). In addition, the siderophore receptor and porin proteins vaccine also lowered the shedding of O157 but not non-O157 (Cull et al. 2012, Cernicchiaro et al. 2014). Recently, the use of probiotics in sheep reduced the non-O157 STEC shedding significantly on 3rd, 5th and 6th days post inoculation

(Rigobelo et al. 2014). Use of probiotics may cause the lower availability of space and nutrition to pathogenic bacteria, consequently reducing their prevalence in feces and on carcass. Supply of clean water and food is essential to maintain healthy cattle herds and to prevent the horizontal transmission of STEC among cattle (Callaway et al. 2013). Fasting and some diets alter the shedding of *E. coli* O157 in cattle; however, the results are not consistent. Switching cattle diet from grain based diet to hay reduces the shedding of STEC O157, but it is not practical before slaughter (Callaway et al. 2009). All these farm management techniques do not eliminate bacteria from cattle but may help to reduce their concentration on farms as well as in slaughterhouses.

STEC (O157 & non-O157) association with insects mainly house flies and stable flies

Even though several studies addressed the association of STEC O157 and house flies, not much is known about the ecology of non-O157 STEC and its relation to house flies. Both house flies and stable flies build very large populations in confined cattle environments because of their larval developmental habitat.

Rahn et al. (1997) in Canada detected VTEC in flies from the dairy cattle environment; however, fly species were not identified. EHEC O157:H7 was identified in house flies for the first time in Japan in 1999 (Iwasa et al. 1999). They detected five STEC positive isolates out of the total 310 flies from a cattle farm. In addition, Moriya et al. (1999) in Japan also reported STEC O157 positive house flies from a nursery school, which was near to a farm (~ 30 m). *Escherichia coli* O157:H7 were detected in the feces of house flies from a dairy farm and were resistant to lincomycin, erythromycin, and ampicillin (Buma et al. 1999). Sasaki et al. (2000) reported that inoculated house flies are able to carry bacteria in their feces at least for 24 h. The

concentration of *E. coli* O157 persisted in the house fly crop for at least 4 days (Sasaki et al. 2000). Another study by Kobayashi et al. (2002) reported the transmission of *E. coli* O157 from inoculated house flies to food (boiled potato and raw beef) through their feces and 10^2-10^5 fold proliferation of bacteria on the food in case of improper storage (29°C) for 24 h was observed. This indicates that a small amount of bacterial excreta from house flies can be sufficient for human infections. Another study by Alam and Zurek (2004) showed that house flies on a cattle farm in the U. S. also carried *E. coli* O157:H7.

The larval development of house flies requires microbial communities (Zurek et al. 2000). Rochon et al. (2004, 2005) studied the larval development of house flies when fed artificially with *E. coli* and observed 62% survival (higher than stable flies, 25%) and bacteria were detected in pupae and emerging adult flies. *Escherichia coli* O157:H7 were found to proliferate in the mouthparts of house flies causing bio-enhanced transmission of bacteria (Kobayashi et al. 1999). Bacteria were detected until day 3 post inoculation in the digestive tract and in the feces of house flies. Detection of bacteria for such a long period may be due to their proliferation in labellum.

House flies were able to transfer nalidixic resistant *E. coli* O157:H7 (Nal^REcO157) to cattle and to their drinking water in the confined cattle environment (Ahmad et al. 2007). *Escherichia coli* were observed up to the concentration of 1.1×10^6 CFU/g of cattle feces when house flies were inoculated with Nal^REcO157 in the concentration of 1.2×10^8 CFU/ml for 48 h. The prevalence of bacteria decreased to 62% on day 19 in feces but observed until day 11 in feces of all calves. Bacteria were also observed sporadically in water given to the calves. The role of house flies in the contamination of leafy green vegetables (spinach) was studied by Talley et al. (2009). GFP tagged *E. coli* O157 were transmitted by house flies to spinach leaves under

the laboratory conditions. The quantitative study of the bacteria that can be carried by house flies in its body surface when exposed to food (milk-sugar solution, steak, and potato salad) inoculated with 10^8 CFU/ml of GFP tagged *E. coli* was studied by De Jesus et al. (2004) and approximately 10^3 CFU/landing was observed (De Jesús et al. 2004). They also enumerated the bacteria from each landing on the sterile surface after the exposure. Highest number of *E. coli* was observed from flies exposed to steak (3.77±1.28 CFU/fly) followed by sugar-milk solution (2.93±1.24) and potato salad (2.25±0.64). *Escherichia coli* were shown to survive and multiply on the spinach leaf and were observed until day 13 of post inoculation (Wasala et al. 2013).

Human incidence with STEC is high during the summer months (CDC 2011) when the outdoor activities such as barbeques and picnics (consumption of improperly cooked meat) is common and is the peak season for flies as well (Mullen and Meyer 1987, Talley et al. 2002). Until now, most of the researches are focused on O157:H7. We have no data available on the association of non O157 STEC and house flies.

Stable flies and STEC: Very little is known about the association of STEC with stable flies and if they play a role in the dissemination of *E. coli*. STEC was not found in stable flies from the nursery school and the confined cattle environment (Moriya et al. 1999, Buma et al. 1999, Szalanski et al. 2004, Keen et al. 2006). The survival of stable flies larva on manure inoculated with *E. coli* alone was poor (25% survival) in comparison to that of house flies (62% survival) (Rochon et al. 2004). In addition, stable fly larvae when fed with *E. coli* did not carry bacteria to teneral adults in contrast to that of house flies (Rochon et al. 2004, 2005).

Recently, one study demonstrated that stable flies from dairy farms in Brazil positive for STEC. Six isolates of STEC were detected from 200 flies (three isolates from body surface, two from intestinal content, and one from mouth parts). STEC were positive for *stx1+stx2* (4), one

carried *stx1* (1), and one with *stx1+stx2+eae* (1). The highest percentage of *E. coli* was isolated from the surface (52.3%) followed mouthparts (31.8%) and the digestive tract (15.9%). Unfortunately, STEC were not serotyped (de Castro et al. 2013).

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Chapter 2 - Prevalence of non-O157 Shiga-toxigenic *Escherichia coli* (STCE) in house flies (*Musca domestica* L.) from confined cattle environments

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are foodborne pathogens responsible for illnesses such as bloody diarrhea, hemorrhagic colitis (HC), and life-threatening hemolytic uremic syndrome (HUS) (Tarr et al. 2005). It is estimated to cause more than 265,000 illnesses each year in the United States due to STEC (Scallan et al. 2011). The major serogroup of STEC for human infection is O157; however, more than 200 serogroups of non-O157 STEC is reported to cause STEC illness (Caprioli et al. 2005, Hedican et al. 2009, Käppeli et al. 2011). Both O157 and non-O157 STEC are associated with multiple outbreaks infecting thousands of people every year globally, mainly affecting children <4 years old (Majowicz et al. 2014). Infection is caused by Shiga-toxins belonging to two major gene families *stx1* and *stx2* along with the intimin protein which is essential for the bacterial attachment to the intestinal epithelium and is encoded by *eae* (Gyles 2007). Treatment of STEC infections using antibiotics is controversial over long time (Carter et al. 1987, Dundas et al. 2001) and mainly includes supportive therapy. Human infections with non-O157 STEC are increasing in recent years (Brooks et al. 2005, Johnson et al. 2006, Gould et al. 2013). Six serogroups of non-O157 STEC (O26, O45, O103, O111, O121, and O145) are responsible for more than 70% of non-O157 human infections (Gloud et al. 2009, CDC 2011) and these serogroups are declared as adulterant in ground beef and nonintact beef products by the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS). In addition, one serogroup of non-O157 STEC, O104 was responsible for a large outbreak in Germany in 2011 resulting in death of 39 people (Frank et al. 2011).

Cattle are the most important asymptomatic reservoirs of STEC O157:H7. Bacteria colonize the rectal anal junction of cattle and are released to the environment via their feces (Karmali et al. 2010). They are also reported to carry non-O157 STEC (Bettelheim 2000, Caprioli et al. 2005, Menrath et al. 2010, Renter et al. 2007). Cattle remain healthy despite of harboring STEC because they lack the vascular receptors (globotriaosylceramide (Gb3) for Shiga toxins (Pruimboom-Brees et al. 2000). STEC have been identified in diverse animals including mammals, birds, fish and insects (Persad and LeJeune 2014). Insects such as house flies (*Musca domestica*) (Iwasa et al. 1999, Alam and Zurek 2004), stable flies (*Stomoxys calcitrans*) (de Castro et al. 2013), dump flies (*Hydrotaea aenescens*) (Szalanski et al. 2004), and dung beetles (*Catharsius molossus*) (Xu et al. 2003) around the animal farms were found carrying STEC O157.

House flies are known as a mechanical vector as they can harbor and disseminate human pathogens (Graczyk et al. 2001). Transmission of pathogens takes place mainly by regurgitation, fecal decomposition, and attachment to the body parts (Graczyk et al. 2001). They can carry pathogens such as *Yersinia pseudotuberculosis* up to 36 h (Zurek et al. 2001), *Aeromonas caviae* up to 8 days (Nayduch et al. 2002), and *Staphylococcus aureus* up to 6 h post infection (Nayduch et al. 2013) in their gut under the laboratory conditions. In addition, Doud and Zurek (2012) showed that *Enterococcus faecalis* can multiply in the crop of the house fly and bacteria can persist in the midgut until 96 h post infection. *Escherichia coli* O157:H7 were found to proliferate in the mouthparts of the house fly causing bio-enhanced transmission of bacteria (Kobayashi et al. 1999). House flies are able to transfer *E. coli* O157:H7 to cattle and their drinking water in confined cattle environment (Ahmad et al. 2007). Moreover, studies showed

that house flies are capable of contamination and transfer of *E. coli* O157:H7 from inoculated food to sterile surfaces and (De Jesús et al. 2004), spinach leaves (Wasala et al. 2013).

Human illnesses with STEC are frequent in summer (CDC 2011) when the human outdoor activities such as barbeques and picnics (consumption of improperly cooked meat) are common. And, this is the peak season for flies in feedlots and dairy farms as well (Lysyk 1993, Mullens and Meyer 1987, Talley et al. 2002). Until now, most of the studies focused on STEC O157:H7 and there is no data available on the association of non-O157 STEC and house flies. The present study aimed to assess the prevalence of seven serogroups of non-O157 STEC (O104, O103, O145, O45, O121, O26, and O111) in house flies from confined cattle environments using culture-based approach followed by molecular analysis.

Materials and Methods

Collection of house flies:

House flies were collected from nine feedlots and three dairy farms in summer and fall 2014 (June to November). Four feedlots in Nebraska, and Texas, and one feedlot in Oklahoma each were visited twice. House flies were also collected from a single dairy farm located in Minnesota, Florida and California during two visits. Flies around the cattle pens and feed bunks were collected using sweep net, kept in ziploc bags, and brought to the laboratory on wet ice within 24 h for further processing.

Isolation and detection of STEC in house flies:

(a) Direct plating:

House flies were identified according to morphology using stereomicroscope. A total of 463 house flies were processed. Out of which, 175, 96 and 48 were from Nebraska, Texas, and Oklahoma, respectively, and 48 each from Minnesota, Florida, and California.

Processing: House flies were surface sterilized using 0.5% sodium hypochlorite, 70% ethanol, and sterile water as described by Zurek et al. (2000) and individually homogenized in 1.0 ml phosphate buffered saline (PBS, MP Biomedicals, Solon). Fly homogenate (100 µl) was spread plated on modified Posse agar (mP). The mP agar was prepared according to the protocol of Possé et al. (2008) with minor modifications in the concentrations of supplements: novobiocin (5.0 mg/L) and potassium tellurite (0.5 mg/L). Colony forming units (CFU) were determined after 24 h incubation at 37°C. Up to 8 phenotypically different colonies were selected from mP and cultured overnight at 37°C on TSA (Tryptic Soy Agar) (Bacto-TSA, Becton Dickinson, Sparks, MD) to obtain fresh cells for PCR detection of non-O157 STEC.

(b) Enrichment:

The house fly homogenate (700 µl) was added to 10 ml of EC broth (Oxoid, Basingstoke, England) and then incubated at 40°C for 6 h at 50 rpm. Based on detectable colony count on mP and turbidity of EC broth, fly samples were subjected to immunomagnetic separation (IMS). (Dynal Biotech, New York) by pooling the serogroup specific IMS beads (Abraxis LLC, Warminister, PA) into two groups, A: serogroups O26+O45+O111+O104 and B: serogroups O103+O145+O121. One ml of the enriched house fly sample was then added to the pooled beads (20 µl of each serogroup) for IMS. A total of two IMS runs were completed for each sample tested. The IMS method was carried out following the manufacturer's instructions (Dynal Biotech, New Hyde Park, NY) and consisted of a 10 min. binding step at the speed dial at 15-25 followed by magnetic capture of 3 min and washing with 1000 µl of Phosphate-buffered saline (PBS) tween 20. Washing step was done twice and elution was carried out with 100 µl of PBS tween 20. Following the IMS, 50 µl of IMS beads suspension was spread plated on mP agar after 100 fold dilutions. After 24 h incubation at 37°C up to 8 phenotypically different colonies were

selected from both group A and B and streaked on TSA and cultured overnight at 37°C to obtain fresh cells for PCR.

Multiplex PCR

Up to 24 colonies (8 from direct plating and 16 from enrichment) were tested for serogroups of interest (STEC-8). Genomic DNA was prepared from 8 pooled cultures from TSA by boiling in a thermocycler for 10 min in 80 µl of autoclaved deionized distilled water. Individual colonies were picked up using the tip of autoclaved toothpick (Great Value, Bentoville, AR) and were suspended in water in 0.2 ml PCR tubes (GeneMate, Bioexpress, Lodi, CA) and were placed on thermocycler (MJ Research PTC 200) for boiling at 95°C for 10 min. Each pooled sample was subjected to serogroup specific 8-plex PCR (including primer sets for O104) following the protocol of Bai *et al*., (2012). Primers for STEC-8 were from (Bai et al. (2012) and for O104 from Paddock et al. (2013). Eight serotypes of *E. coli*: JB1-95 O111:H-, CDC 96-3285 O45:H2, CDC 90-3128 O103:H2, CDC 97-3068 O121:H19, 83-75 O145:NM, H30 O26:H11, ATCC BAA-2326 O104:H4, Salami 380-94 O157:H7 were used as positive controls. The PCR procedure included 5 min of denaturation at 94°C followed by 30 cycles at 94°C for 30 s and 67°C for 80 s. The PCR products were run on 1.2% of agarose gel (Amresco, Solon, OH) and were visualized by Gel Doc XR + imaging system under UV light (Bio-Rad, universal hood, Segrate, Italy) using Quantity One (software). The pooled positive samples were further tested individually for confirmation at serogroup-level by single PCR. Genomic DNA for single isolate was prepared in the same way as for pooled samples in 50 µl deionized distilled water. Further, serogroup-positive individual isolates were screened for the virulence genes: Shiga-toxins (*stx1* and *stx2*), intimin (*eae*), and hemolysin (*ehxA*) using 4-plex PCR (Bai et al. 2012).

Statistical Analysis: One way ANOVA was performed to compare the CFU counts on mP in house flies from feedlots and dairies of six states ($P < 0.05$) using Origin 7 (OriginLab, Northampton, MA).

Results

In this study the prevalence of STEC-7 in house flies from cattle feedlots and dairy farms in six states of the US was assessed. Of 463 house flies, 159 (34.3%) were positive for *E. coli* serogroups of interest. None of the flies were positive for the serogroup O111. Of 159 serogroup positive flies, 7 (1.5%) carried the *stx1* gene. Nine isolates possessed *stx1*, *eae*, and *ehxA* gene together (Table 2.1). Moreover, we got two isolates positive for *E. coli* O157 from Oklahoma. The CFU counts on mP agar in house flies from Florida and Oklahoma was significantly different (P = 0.0001) from that of the other states (Nebraska, Minnesota, and California) (Figure 2.3).

Feedlots

Nebraska: A total of 175 house flies were processed from four different feedlots in Nebraska. Of those, 156 (89.1%) were positive for CFU counts on mP agar with a concentration ranging from 1.0×10^{1} to 6.8×10^{6} (mean: $2.1 \pm 0.64\times10^{5}$) CFU/fly (Figure 2.3). The most prevalent serogroup was O104 (48.9.0%) followed by O103 (46.7%), O45 (6.7%), O121 (6.7%), O26 (4.4%), and O145 (2.2%) (Figure 2.1) and, 45 (25.7%) were positive for at least one serogroup of *E. coli* of interest. Moreover, of 45 serogroup positive flies, 7 (15.6%) carried multiple serogroups (Table 2.3). Four O104 positive isolates from one fly obtained by direct plating harbored the *stx1* and *ehxA* genes (Table 2.1). Three O103 positive isolates from three flies also carried the *ehxA* gene but no *stx* (Table 2.2).

Texas: Almost all the house flies (94/96, 97.9%) were positive for CFU counts on mP agar and the concentration ranged between 1.0×10^{1} and 7.8×10^{6} with the mean count of $1.1 \pm 0.15\times10^{6}$ CFU/fly (Figure 2.3). Of 96 house flies, 37 (38.5%) were positive for at least one serogroup and 3/37 (8.1%) were positive for multiple serogroups (Table 2.3). One O103 positive isolate was positive for *stx1*, *eae* and *ehxA* (Table 2.1). The most prevalent serogroup isolated was O104 (83.8%) and followed by O45 (10.8%), O103 (8.1%) and O26 (5.4%) (Figure 2.1). Three O45 positive isolates from two flies also carried the *ehxA* gene but not the *stx* (Table 2.2).

Oklahoma: Almost all the house flies (47/48, 97.9%) tested for STEC were positive for CFU counts on mP agar. The concentration of bacteria ranged from 1.0×10^{1} to 2.2×10^{7} with the mean count of $3.6\times10^{6} \pm 7.6\times10^{5}$. Of 48 house flies, 15 (31.3%) were positive for at least one serogroup and 3/15 (20.0%) were positive for multiple serogroups. The serogroup O104 was the most prevalent (53.3%) followed by O45 (46.6%), O103 (13.3%) and O26 (6.7%) (Figure 2.1). We also got 2, O157 positive isolates (one was positive for O45 as well) from 2 flies (12.5%) by mPCR, but were negative for the virulence genes tested. A total of 13 isolates from 5 flies (10.4%, 5/48) possessed *stx1* (Table 1). Four O103 isolates form 1 fly harbored *stx1*, *ehxA*, (Table 2.1) and nine O45 isolates from 5 house flies possessed *stx1*, *eae* and *ehxA* (Table 2.2).

STEC prevalence in feedlots

A total of 319 house flies were screened from nine feedlots out of which, 297 (93.1%) carried bacteria on mP agar which were presumably non-O157 *E. coli* with some other enterics (*Enterobacter spp.* and *Proteus spp*.). The CFU counts on mP agar ranged between 1.0×10^{1} and 2.2×10^{7} /fly. Serogroup-specific multiplex PCR confirmed that 97/319 (30.4%) house flies were positive for at least one serogroup of interest and 13 flies carried more than one serogroup. A total of 18 isolates from 7 flies were STEC and presented 2.2% (7/319) prevalence. Nine O45

isolates from four house flies carried *stx1*, *eae* and *ehxA* gene. However, five O103 positive isolates from two flies and four O104 positive isolates from one fly carried *stx1* and *ehxA* (Table 2.1).

Dairy Farms

Minnesota: All 48 house flies screened for STEC were positive for CFU counts on mP agar. The concentration of bacteria ranged from 1.8×10^{2} to 1.2×10^{7} with a mean of $5.0 \pm 2.6\times10^{5}$ CFU/fly. Of 48 flies, 20.8% were positive for at least one serogroup. *E. coli* O104 was the most prevalent (50.0%) followed by O103 (30.0%), O26 (20.0%), O121 (10.0%), and O145 (10.0%) (Figure 2.2) and 2/10 (20.0%) carried multiple serogroups; however, no isolates were positive for Shiga-toxin genes and, one O145 isolate carried *eae* and *ehxA* (Table 2.2).

Florida: Almost all the flies (47/48, 97.9%) carried bacteria on mP agar. The CFU counts on mP agar ranged between 2.0×10^{1} and 7.0×10^{7} with a mean count of $6.6 \pm 1.9\times10^{6}$ /fly. Of 48 house flies 83.3% were positive for at least one serogroup and (42.5%) 17/40 flies carried multiple serogroups. However, none of the serogroup positive isolates carried Shiga toxin genes. *Escherichia coli* O121 was the most prevalent (62.5%) serogroup followed by O103 (37.5%), O104 (17.5%), O45 (20.0%), O26 (7.5%), and O145 (2.5%) (Figure 2.2). Six O103 positive isolates from four flies also carried the *ehxA* gene (Table 2.2).

California: Of 48 house flies, 95.8% (46/48) were positive for CFU counts on mP agar. The concentration of bacteria ranged from 4.0×10^{1} to 7.0×10^{6} with a mean count of $4.1 \pm 1.8\times10^{5}$ CFU/fly. Serogroup O145 was the most prevalent (41.7%) followed by O104 (33.3%), O121 (25.0%), O45 (8.3%) and O26 (8.3%) (Figure 2.2). Of 48 flies 25.0% (12) were positive for at least one serogroup of interest and 16.7% (2/12) harbored multiple serogroups (Table 2.3). Six isolates positive for O145 from four flies harbored *eae* and *ehxA* but not *stx* (Table 2.2).

STEC prevalence in dairy farms

A total of 144 house flies from three dairy farms were screened for STEC and (141, 97.9%) were positive for CFU counts on mP agar. The concentration of bacteria ranged from 2.0×10^{1} to 7.0×10^{7} CFU/fly. 8-plex PCR results confirmed that 62 flies were positive for at least one serogroup of interest accounting for the prevalence of 43.1% and 21 flies were positive for more than one serogroup. Six O103 positive isolates from four flies harbored the *ehxA* gene while seven O145 positive isolates from five flies carried *eae* and *ehxA* (Table 2.2). None of the serogroup positive isolates carried Shiga toxin genes (*stx1* and *stx2*).

Discussion

Overall CFU counts on mP agar in this study ranged from 2.1×10^{5} to 6.6×10^{7}. This is similar to the concentration of enterics from various studies (Alam and Zurek 2004, Ghosh and Zurek 2015). Our results showed that 7/463 (1.5%) house flies from confined cattle environments carried non-O157 STEC which is similar to the prevalence of STEC O157:H7 in house flies from cattle farms as reported by others: 5/310, 1.6% (Iwasa et al. 1999), 2.9% from the feed bunk, and 1.4% from the cattle feed storage shed (Alam and Zurek 2004). However, Moriya et al. (1999) reported (5/89, 5.6%) STEC O157:H7 positive house flies from the nursery school in Japan, which was near the cattle farm (~30m). Rahn et al. (1997) also reported 12.5% of flies from dairy cattle positive for STEC O157:H7; however, fly species were not identified.

Many studies focused on association of STEC O157 and house flies; however, none examined non-O157 STEC. Non-O157 STEC has been detected in cattle feces from cattle farms in the U.S. and other parts of the world (Hornitzky et al. 2002, Renter et al. 2007, Musa et al. 2013, Cernicchiaro et al. 2013, Bibbal et al. 2015). The prevalence of *E. coli* O157 in feces of cattle showed strong seasonal variation and was higher during summer than in winter (Barkocy-

Gallagher et al. 2003, Edrington et al. 2006, Ferens and Hovde 2011). In contrast the seasonal prevalence of non-O157 in cattle is not well established (Barkocy-Gallagher et al. 2003). In a recent study, Dewsbury et al. (2015) reported the low (0.8-1.6%) prevalence of non-O157 STEC in summer and was not detected in winter. The density of house flies in confined cattle environment (feedlots or dairy farms) also varied with change in the season and is remarkably high during summer months (Mullens and Meyer 1987, Talley et al. 2002). In addition, incidences of human infections with STEC are higher during summer (CDC 2011). House flies inoculated with *E. coli* O157 were able to transfer bacteria to cattle and their water (Ahmad et al. 2007). Thus, presence of non-O157 STEC positive house flies in confined cattle environments clearly points out the possible role of house flies in dissemination of bacteria to cattle as well as their food and water.

Fleming et al. (2014) investigated the response of the immune effector molecule after the ingestion of GFP expressing *E. coli* O157 by house flies and up regulation of antimicrobial peptides (AMPs) and lysozyme gene expression was observed. Steady decrease in the *E. coli* O157 in the intestinal tract was observed and flies carried O157 at least for 12 h. In our study we surface sterilized the flies before processing. Thus, STEC recovered from house flies were from the internal parts (digestive tract) of flies but not from the body surface, which indicates that house flies may also harbor non-O157 STEC for considerable time in their digestive tract similar to STEC O157. In addition, vector competence data of house flies for STEC-7 from our study (unpublished data) also support this notion. Flies were shown to harbor non-O157 STEC at least for six days post inoculation.

There has been extensive research on STEC O157 in beef cattle environment due to the illness associated with consumption of contaminated beef products (Elder et al. 2000, Keen and

Elder 2002, Williams et al. 2010). Both O157 non-O157 STEC have been reported in the fecal sample from dairy farms as well (Wells et al. 1991, Rahn et al. 1997, Fernández et al. 2010, Lynch et al. 2012, Kang et al. 2014). There are few studies comparing the prevalence of STEC O157 in fecal sample between beef and dairy cattle (Hancock et al. 1994, Sasaki et al. 2013) and results showed higher (0.71-6.4%) STEC prevalence in beef than in dairy cattle (0- 0.28%) which is similar to our results for non-O157 STEC (0 in dairy and 2.2% in feedlots) from house flies. However, Cobbold et al. (2004) reported higher STEC prevalence in dairy cattle (8%) than from feedlot (3%) in feces. Season, age, and diet may affect the shedding of non-O157 STEC in feces of cattle and that may affect the STEC dissemination by house flies whose larval development takes place primarily in cattle manure.

We could not detect Shiga-toxin genes in house flies from dairy farms; however, 43.1% of house flies were positive for non-O157 *E. coli* and 13 isolates carried virulence genes other than *stx* (*eae* and *ehxA)* (Table 2.2) indicating their capacity to harbor virulence genes. Serogroup O104, O103, and O45 were the most prevalent in house flies from feedlots whereas O121, O145, and O104 were prevalent in house flies from dairy farms in FL, CA, and MN, respectively showing variability in the distribution on non-O157 *E. coli.* Serogroup O111 was reported to be least prevalent in fecal samples analyzed from feedlots and dairy farms which may be the reason why we did not get any O111 positive house flies from both feedlots and dairies in our study (Lynch et al. 2012, Cernicchiaro et al. 2013, Dewsbury et al. 2015).

Overall 34.3% of non-O157 *E. coli* serogroup-positive house flies represent very high number in confined cattle environments and there are incidences of human infection with *stx* negative *E. coli* (Friedrich et al. 2007, Bielaszewska et al. 2007, Bielaszewska et al. 2008). The rapid acquisition or loss of virulence genes takes place in *E. coli* because of high genomic

plasticity, and virulence genes are located in the mobile elements (phages, pathogenicity islands, plasmids) (Bielaszewska et al. 2007, Ison et al. 2015). The majority of O26 serogroup positive isolates recovered from patients harbored the *stx1* gene before 1994; however, in later years there was a striking shift of *stx* genotype from *stx1* to *stx2* (Zhang et al. 2000) indicating the shifting of virulence gene profile. The relatedness of Shiga-toxin negative and intimin positive O26 isolates from cattle and human isolates were studied by Ison et al. (2015) and results showed the possibility of *stx* negative strains may have previously contained a prophage carrying *stx* or could acquire this prophage. We also found seven O145 positive *E. coli* isolates from 5 flies carrying *eae* and *ehxA* genes and another 12 isolates from nine flies positive for *ehxA* (Table 2.2).

Out of 18 STEC isolates from seven flies, 50% harbored *stx1* along with *eae* and *ehxA* gene, while other 50% were positive for *stx1* and *ehxA* gene and lacked *eae*. We did not get any isolates with *stx2* gene. Most of the human infection of HUS is associated with *stx2* (Friedrich et al. 2007, Boerlin et al. 1999, Griffin and Tauxe 1991, Heuvelink et al. 1995, Kawano et al. 2008), however, recently non-O157 STEC with *stx1* alone were shown to produce infection in similar frequency as *stx2* (Käppeli et al. 2011, Bekal et al. 2014). Moreover *eae* is also not essential for infection as number of outbreaks with non-O157 STEC serogroups lacked *eae* (Paton and Paton 1998, Frank et al. 2011).

A total of 7.3% (34) of house flies were positive for more than one non-O157 *E. coli* serogroups in our study similar to fecal and beef samples from cattle (Wells et al. 1991, Cernicchiaro et al. 2013, Wasilenko et al. 2014). Interestingly, we identified STEC positive as well as negative multiple serogroups of non-O157 *E. coli* from the same house fly sample indicating the potential of horizontal transfer of virulence genes between bacteria in the gut of fly. Non-O157 *E. coli* positive flies with multiple serogroups did not harbor Shiga-toxin genes

except for one fly from Texas that was positive for STEC O103 with *stx1* and *ehxA* and was also positive for O26. Further research is essential to elucidate the exact role of house flies in the ecology of non-O157 STEC and their association with human illnesses.

Conclusion

In conclusion, this study demonstrated that house flies from confined cattle environments carried non-O157 STEC and thus may play an important role in the ecology and dissemination of these pathogenic bacteria among individual cattle and possibly to the surrounding environment. Controlling house fly population by manure management should be an integral part of the control of dissemination of STEC within confined cattle environments as well as surrounding environments.

Tables and Figures

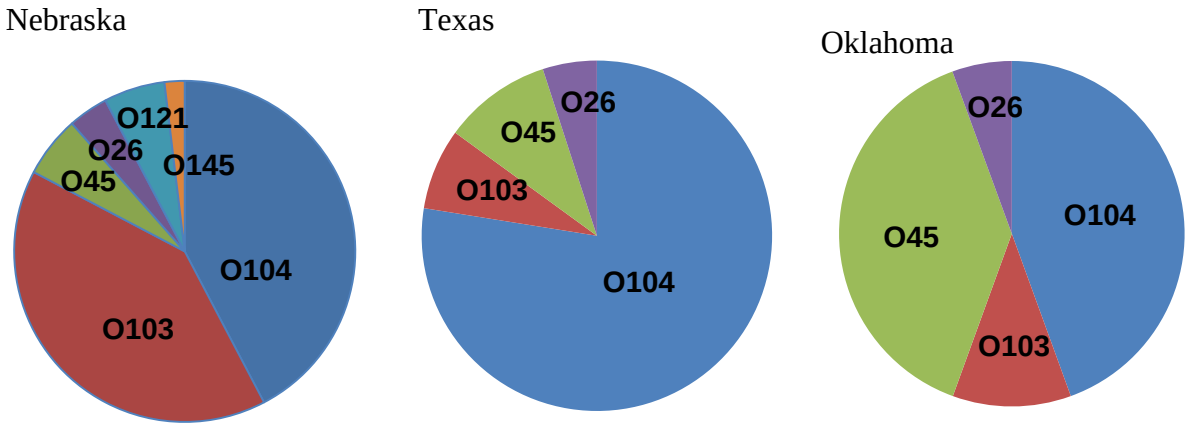

Figure 2.1 Distribution of serogroup positive house flies in feedlots of Nebraska (NE), Texas (TX) and Oklahoma (OK).

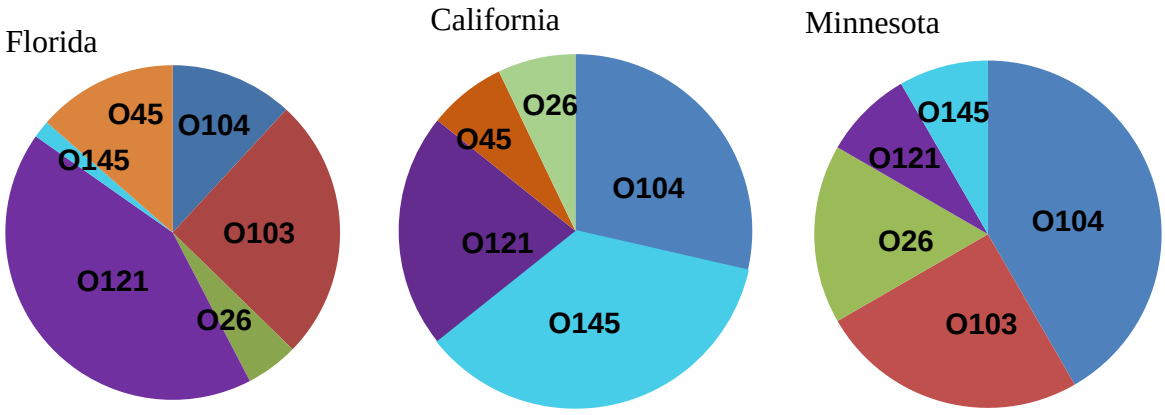

Figure 2.2 Distribution of serogroup positive house flies in dairy farms of Florida (FL), California (CA), and Minnesota (MN).

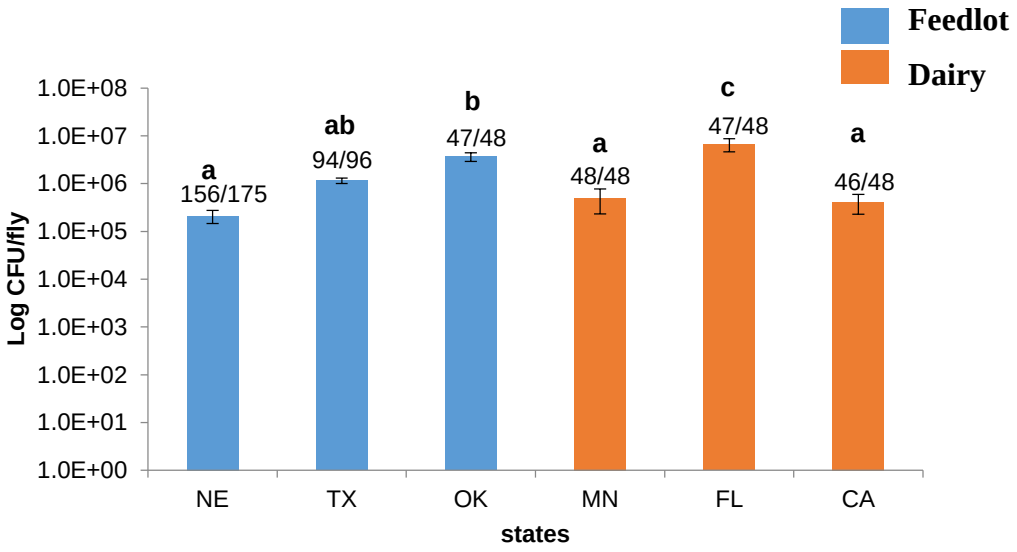

Figure 2.3 Total CFU counts on mP agar associated with house flies from feedlots and dairies of six states.

Number above the bar represents the number of house flies with detectable colony count on mP agar following direct plating technique /total number of flies processed from each state. Different letters above each bar indicate significant differences ($P < 0.05$) among CFU counts on mP agar from different states. Error bars are standard of mean.

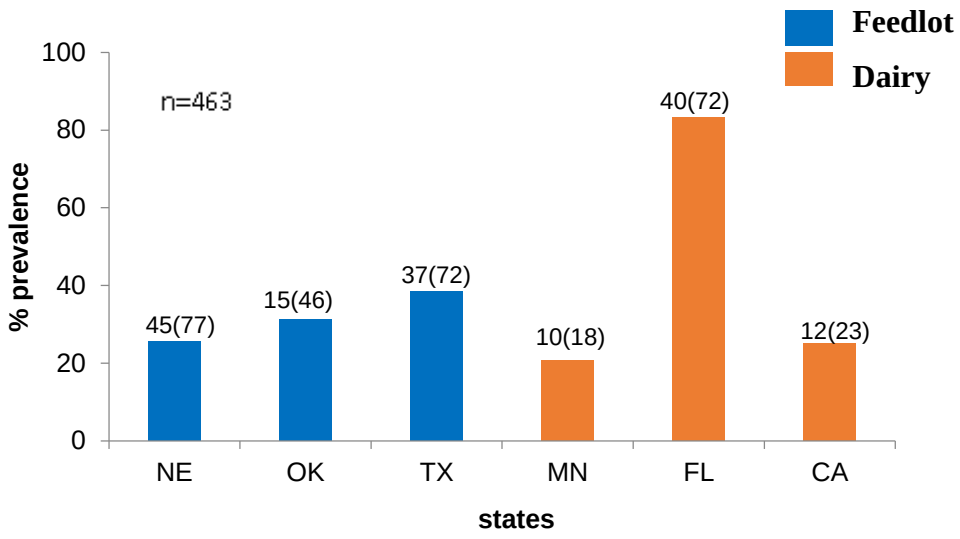

Figure 2.4 Prevalence of non-O157 *E. coli* in house flies from feedlots and dairies of six states. Number above the bar represents the number of serogroup positive flies followed by number of positive isolates from each state in parenthesis and "n" refers to the total no. of house flies processed.

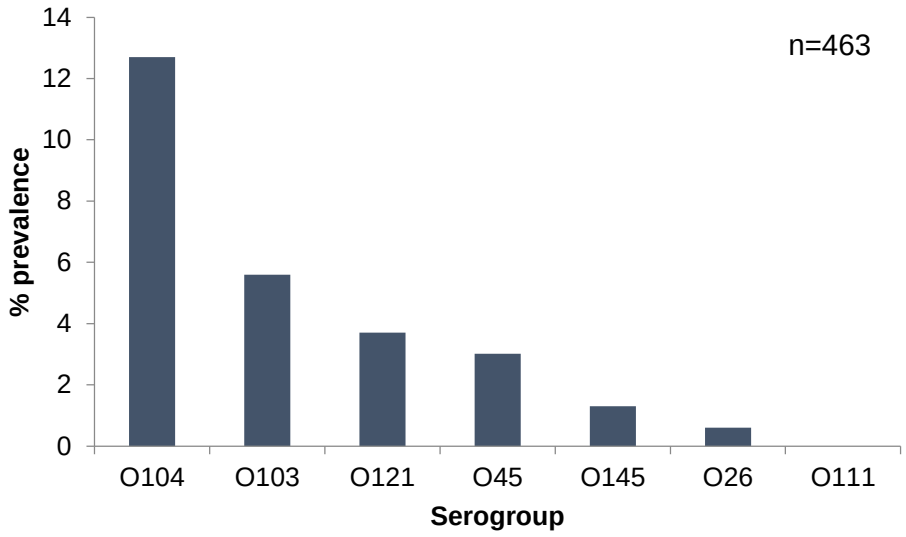

Figure 2.5 Prevalence of house flies with non-O157 *E. coli*.
"n" stands for the total no. of house flies processed.

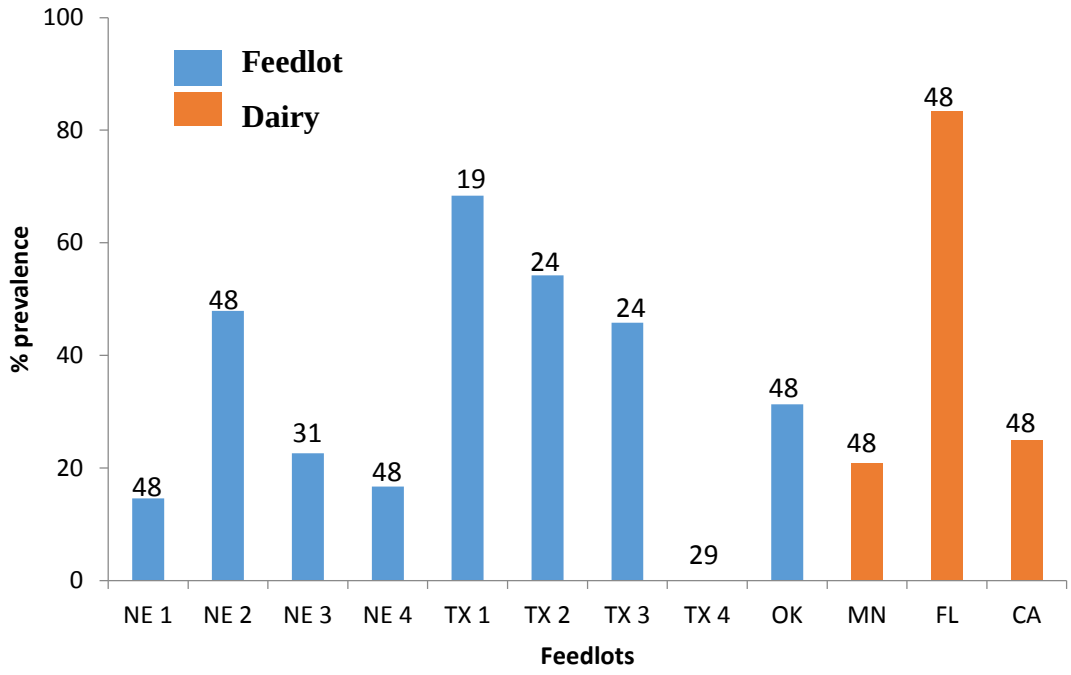

Figure 2.6 Prevalence of non-O157 *E. coli* in house flies from nine feedlots and three dairy farms.

Number above the bar represents the no. of house flies processed from each feedlot. All the house flies except from one feedlot in Texas (TX4) carried non-O157 *E. coli*.

Table 2.1 Detailed information on STEC isolates carried by house flies.

State	Serogroup	No. of house flies (no. of isolates)	Virulence Profile
OK	O103	1(4)	*stx1*, *ehxA*
	O45	4(9)	*stx1*, *eae*, *ehxA*
TX	O103	1(1)	*stx1*, *ehxA*
NE	O104	1(4)	*stx1*, *ehxA*

Table 2.2 Detailed information on non-O157 *E. coli* positive isolates having virulence genes other than the Shiga-toxin.

State	Serogroup	No. of house flies (no. of isolates)	Virulence Profile
TX	O45	2(3)	*ehxA*
NE	O103	3(3)	*ehxA*
MN	O145	1(1)	*eae*, *ehxA*
FL	O103	4(6)	*ehxA*
CA	O145	4(6)	*eae*, *ehxA*

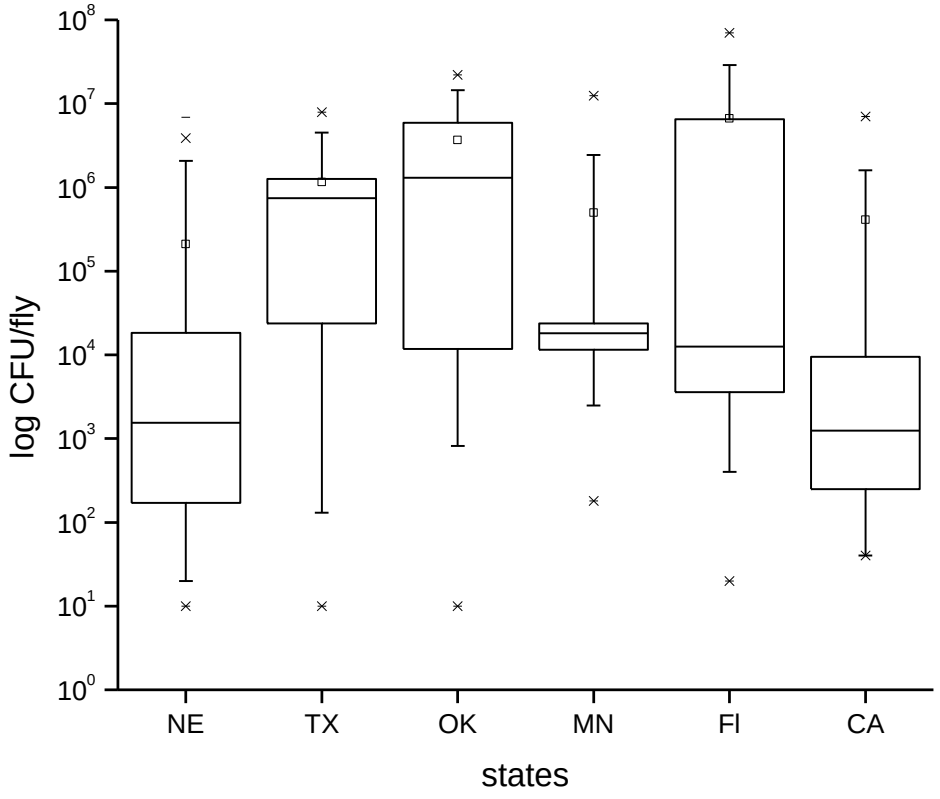

Figure 2.7 Boxplot showing CFU counts on mP agar in house flies from six states.

Table 2.3 Prevalence of non-O157 *E. coli* in house flies from six states.

State	No. of house flies / +ve (%)	Serogroup	No. of house flies (no. of isolates)
NE	175/45 (25.7)	O104	17(39)
		O103	16(22)
		O145	1(1)
		O121	2(2)
		O45	2(5)
		O104+O26	1(1)
		O103+O104	4(5)
		O45+O103	1(1)
		O26+O121	1(1)
		-	45(77)
TX	96/37(38.5)	O104	29(60)
		O103	1(3)
		O45	4(6)
		O104+O26	1(1)
		O103+O104	1(1)
		O26+O103	1(1)
		-	37(72)
OK	48/15(31.25)	O104	6(22)
		O103	1(4)
		O45	4(14)
		O157	1 (1)
		O104+O45	2(2)
		O103+O26	1(1)
		O45+O157	1 (4)
		-	16(48)
MN	48/10(20.8)	O104	3(6)
		O103	2(2)
		O145	1 (1)
		O121	1(1)
		O26	2(6)
		O103+O104	1(1)

		O104+O145	1(1)
		-	10(18)
FL	48/40(83.3)	O104	1(9)
		O103	6(15)
		O145	1(1)
		O121	12(20)
		O45	2(4)
		O26	1(1)
		O104+O45+O121	2(3)
		O104+O103+O121	1(1)
		O104+O26	1(1)
		O104+O45	1(1)
		O121+O26	1(3)
		O103+O45	1(2)
		O121+O103	6(6)
		O121+O104	1(1)
		O121+O45	2(2)
		O104+O103	1(2)
		-	40(72)
CA	48/12(25)	O104	4(11)
		O145	5(6)
		O121	2(2)
		O45	1(2)
		O145+O121	1(1)
		O104+O26	1(1)
			12(23)
Total	159/463(34.3%)		160(310)

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Chapter 3 - Prevalence of eight serogroups of Shiga-toxigenic *Escherichia coli* (STEC) in stable flies (*Stomoxys calcitrans* L.) from a confined beef cattle

Introduction

Beef and dairy cattle are asymptomatic reservoirs of Shiga-toxigenic *Escherichia coli* (STEC), important human foodborne pathogens that are released to the environment in cattle feces (Karmali et al. 2010). STEC causes bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Tarr et al. 2005) affecting~ 250,000 people every year in the U. S. (Scallan et al. 2011). Young children (<4 years old), elderly adults and immunocompromised individuals are at high risk and tend to be affected severely by these bacteria (Majowicz et al. 2014). Infectious dose of STEC O157 is very low (10-100 cells) and even lower than 10 cells are reported to cause an infection (Hara-Kudo and Takatori 2011). STEC O157 is the main cause of human illness; however, in the recent years more than 200 serogroups of non-O157 STEC are reported to cause infections (Caprioli et al. 2005, Hedican et al. 2009, Käppeli et al. 2011). More than 70% of non-O157 infection is caused by six serogroups of STEC called "big six" serogroups and include O26, O45, O103, O111, O121, and O145 (Gloud et al. 2009, CDC 2011). In addition, these serogroups are declared as adulterant in ground beef and non-intact beef products by the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS). Another non-O157 serogroup, O104 is the hybrid strain of EAEC and STEC, was responsible for a large outbreak in Germany in summer 2011 killing 39 people (Frank et al. 2011).

Stable flies (*Stomoxys calcitrans* L.) are blood-feeding insects, both male and female ingest blood from animals including cattle especially from front legs and lower abdomen. The painful bites lower the cattle weight gain and milk production (Campbell et al. 2001). Stable flies

develop in animal manure and build up very large populations around confined and pastured cattle, and cause the U.S. cattle industry about $2.2 billion in economic losses annually (Taylor et al. 2012). Stable fly larval survival and development depend on an active bacterial community in animal manure (Romero et al. 2006). Rochon et al. (2004, 2005) showed in laboratory bioassays that, stable fly larvae do not develop well on *E. coli* monocultures in contrast to house flies (another fly species common around confined cattle). *Escherichia coli* are ingested but not readily digested by stable fly larvae and are retained during pupation. Adult stable flies may acquire STEC from the cattle environment by contact and during feeding from the contaminated cattle skin. The vector competence of stable flies was assessed under laboratory condition for *Cronobacter (Enterobacter) sakazakii* and West Nile virus (Mramba et al. 2007, Doyle et al. 2011). Stable flies were able to transmit West Nile virus mechanically until 6 h (especially first hour of infection). In addition, *Cronobacter sakazaki* were also carried by stable flies until day 20 post inoculation indicating their role as a vector of bacteria (Mramba et al. 2007) and were able to contaminate food source (blood, sugar water). However, the prevalence of *C. sakazaki* was reduced to 55% at the end of the experiment. Several studies done in the past shown that stable flies can act as a mechanical vector of pathogens (viruses and bacteria) (reviewed in Baldacchino et al. 2013) for several days in the laboratory conditions but no study has reported the transmission of pathogens by stable flies in nature.

Previous studies showed that house flies carried STEC O157:H7 in a cattle feedlot and were able to transfer these bacteria to cattle and their drinking water (Alam and Zurek 2004, Ahmad et al. 2007). However, very little is known about potential role of stable flies in the ecology of STEC. In addition, STEC O157 shedding by cattle shows strong seasonal prevalence and peaked at summer which corresponds with the flies population in feedlots (Lysyk 1993,

Mullens and Meyer 1987, Talley et al. 2002); however, the shedding of non-O157 by cattle is not well understood (Barkocy-Gallagher et al. 2003, Dewsbury et al. 2015).

Recently, a study from Brazil identified STEC in stable flies collected from dairy farms (de Castro et al. 2013). However, the authors did not serotype the isolates, and did not indicate the prevalence of stable flies positive for STEC. They showed that out of 44 *E. coli*, six isolates were STEC (four positive for *stx1+stx2*, one carried *stx1*, and one with *stx1+stx2+eae*). In the present study, we assessed the prevalence of eight serogroups of STEC in stable flies collected from a commercial cattle feedlot using culture-based approach followed by multiplex PCR.

Materials and Methods

Collection of stable flies:

Stable flies (n = 50) were collected on a weekly basis for twelve weeks from June 9 to August 25, from a commercial feedlot in central Nebraska. Flies were collected using a sweep net, placed in ziploc bags, and shipped overnight on wet ice to our laboratory.

Isolation and detection of STEC in stable flies:

a) Direct plating:

Stable flies were identified by morphology using stereomicroscope. Flies were then surface sterilized using 0.05% sodium hypochlorite and 70% ethanol (Zurek et al. 2000) to eliminate cross-contamination during collection. Fifteen randomly selected flies from each collection were individually homogenized in 1.0 ml phosphate buffered saline (PBS, pH 7.2) (MP Biomedicals, Solon, OH) and serially diluted in PBS. One hundred microliters of fly homogenate was spread plated on sorbitol MacConkey agar (Difco-SMAC, Becton Dickinson, Sparks, MD) supplemented with cefixime (25 µg/l) and potassium tellurite (1.25 mg/l) (CT-SMAC) and on modified Posse agar (mP). The mP agar was prepared according to the protocol

of Possé et al. (2008) with minor modifications in the concentrations of supplements: novobiocin (5.0 mg/l) and potassium tellurite (0.5 mg/l). Colony forming units (CFU) were counted on each medium after 24 h incubation at 37°C. For detection of *E. coli* O157:H7, non-sorbitol fermenting (colorless) colonies from CT-SMAC were screened by the latex agglutination test for the O157 antigen (Oxoid, Basingstoke, England). For non-O157 serogroups, up to 6 phenotypically different colonies were selected from mP agar and streaked on Tryptic Soy Agar (TSA) (Bacto-TSA, Becton Dickinson, Sparks, MD), and cultured overnight at 37°C to obtain fresh cells for PCR.

b) Enrichment:

Seven hundred µl of the fly homogenate was added to 10 ml of EC broth (Oxoid, Basingstoke, England) and incubated on a shaker at 40°C for 6 h at 50 rpm. Based on colony count from direct plating on mP and CT-SMAC, fly samples with ≥20 CFU/fly on mP and/or CT-SMAC were subjected to immunomagnetic separation (IMS) following the manufacturer's instructions (Dynal Biotech, New Hyde Park, NY). Enriched samples were pooled into two groups (group A: serogroups O103+O104+O26 and group B: serogroups O145+O45+O121+O111), processed, then 100 fold diluted, and 100 µl spread plated on mP agar. After 24 h incubation at 37°C, up to six phenotypically distinct colonies were selected from each group and streaked on TSA and cultured overnight at 37°C for PCR.

Multiplex PCR

Up to 18 colonies (6 from direct plating and 2×6 from enrichment/IMS) per fly were tested for STEC-8. Genomic DNA was prepared from six pooled colonies from TSA by boiling in a thermocycler for 10 min. in 50µl of deionized autoclaved water. Individual colonies were picked up using the tip of autoclaved toothpick (Great Value, Bentoville, AR) and were

suspended in water in 0.2 ml PCR tubes (GeneMate, Bioexpress, Lodi, CA) and were placed on thermocycler (MJ Research PTC 200) for boiling at 95°C for 10 min. Each pooled sample was subjected to serogroup-specific 8-plex PCR following the protocol of (Bai et al. 2012). Primers for STEC-7 were from Bai *et al.* (2012) and for O104 from Paddock et al. (2013). The PCR procedure included 5 min of denaturation at 94°C followed by 30 cycles at 94°C for 30 s and 67°C for 80 s. The PCR products were run on 1.2% of agarose gel (Amresco, Solon, OH) and were visualized by Gel Doc XR + imaging system under UV light (Bio-Rad, universal hood, Segrate, Italy) using quantity one (software). Positive samples were then further screened by testing individual bacterial isolates for serogroup confirmation by single PCR. Serogroup-positive individual isolates were screened for virulence genes *stx1* and *stx2* (Shiga-toxins), *eae* (intimin), and *ehxA* (hemolysin) using 4-plex PCR (Bai et al. 2012). Eight serotypes of *E. coli*: JB1-95 O111:H-, CDC 96-3285 O45:H2, CDC 90-3128 O103:H2, CDC 97-3068 O12:H19, 83-75 O145:NM, H30 O26:H11, ATCC BAA-2326 O104:H4, Salami 380-94 O157:H7 were used as positive controls.

Statistical Analysis: Correlation between prevalence of enterics (non-O157 *E. coli*, *Enterobacter spp*., and *Proteus spp*., that grow on mP agar) positive flies and bacterial concentration on mP agar was assessed by the multiple regression analysis ($P < 0.05$). One-way ANOVA was performed to compare the CFU counts over the twelve week period on each of two different culture media (mP and CT-SMAC) ($P < 0.05$) in Origin 7 (OriginLab, Northampton, MA).

Results and Discussion

Of 180 stable flies, 67 (37.2%) were positive for bacteria on mP agar (designed to culture and differentiate non-O157 serogroups and allows the growth of other enterics- *Enterobacter*

spp. and *Proteus spp*.) and 55 (30.5%) were positive for bacteria on CT-SMAC (selective for enterics and differentiating *E. coli* O157). The CFU counts ranged from 1.0×10^{1} to 3.2×10^{7} (mean: $3.6\pm1.05\times10^{6}$) /fly on mP and 1.0×10^{1} to 6.0×10^{5} (mean: $1.2\pm1.08\times10^{4}$) CFU/fly on CT-SMAC (Figure 3.1). Interestingly, the weekly prevalence of positive flies peaked (86.6%) in the last week of sampling and corresponded to the highest CFU counts (mean: $1.7\pm0.32\times10^{7}$ /fly) (Figure 3.2). A moderate correlation was found between the prevalence of enteric positive flies and the bacterial concentration (R^2=0.509). There was no significant difference in enteric concentrations on CT-SMAC over the twelve week period (P = 0.856); however, a significant difference (P = 0.001) was observed in CFU counts on mP agar between the week 12 and all other weeks with exception of week 4 and week 10 (Figure 3.1).

Previously, our group collected stable flies from the pastured and confined cattle environment and reported 44.3% (411 out of 982) flies carried enterics with a mean concentration of 6.4×10^{4} CFU/fly (Mramba et al. 2006). We also found 12.9% (120/928) of stable flies carried fecal coliforms with a mean concentration of 8.7×10^{3} CFU/fly (Mramba et al. 2006). In contrast to stable flies, the majority (95.4%) of house flies collected from a beef cattle feedlot carried fecal coliforms with the mean concentration 2.1×10^{5} CFU/fly (Alam and Zurek 2004).

All sorbitol-negative colonies on CT-SMAC tested negative for the O157 antigen. Selected colonies from mP agar were further screened using multiplex PCR to detect STEC-8. Of 180 stable flies, only 2 flies were positive for the serogroups of interest: O45 (1 isolate) and O26 (2 isolates), all from the enrichment/IMS approach and neither of them carried the virulence genes tested. In a parallel study, cattle feces were collected from the same feedlot during the same collection period and screened for the presence of STEC following the same approach

(Dewsbury et al. 2015). Almost 60% of fecal samples were positive for *E. coli* serogroup O103, followed by O157 (43.1%), O26 (22.0%), O45 (16.5%), O145 (3.2%), O121 (2.1%), and O111 (0.2%). They also showed that serogroups O157, O26, O103, and O45 carried the *stx1* and/or *stx2* and *eae* genes (Dewsbury et al. 2015). Overall, our current data indicate that adult stable flies do not carry STEC-8 in the digestive tract despite presence of STEC, especially *E. coli* O157, in cattle feces on the same farm. This data is in agreement with the previous reports of (Rochon et al. 2004, Rochon et al. 2005) where they suggested, based on laboratory bioassays that stable fly larvae and pupae retain and accumulate *E. coli* in the gut; however, most teneral adults were bacteria free. Their bioassays also showed that survival and retention of *E. coli* throughout various life stages of stable flies was significantly lower compared to that of house flies (Rochon et al. 2004, 2005). *Escherichia coli* O157 were not detected in stable flies in other studies as well (Moriya et al. 1999, Szalanski et al. 2004).

Shiga-toxigenic *E. coli* was detected in stable flies from dairy farms in only one study and bacteria were isolated from body surface, mouthparts, and abdominal digestive tract (de Castro et al. 2013). The highest percentage of *E. coli* were isolated from body surface (52.3%) followed mouthparts (31.8%) and digestive tract (15.9%). Six isolates of STEC were detected from 200 flies (three were from body surface, two from intestinal content and one from mouthparts) (de Castro et al. 2013). We surface sterilized the flies before processing and thus bacteria were from the gut only.

Adult blood-feeding flies secrete salivary compounds to evade host defense response. These compounds include complex mixtures of serine proteases, endonucleases, anti-thrombins, antimicrobial peptides (Wang et al. 2009), and may help in digestion of microbes during their passage through the crop and intestinal tract of the fly. Therefore, based on the previous

laboratory bioassays data and the field data collected in this study, we conclude that, unlike house flies, stable flies do not play a major role as a vector of STEC.

Conclusion

Results from our study reveal that stable flies unlike house flies do not play a role in dissemination of STEC in feedlots and dairies even though they are highly important economic pest in the United States because of their painful bites.

Figures

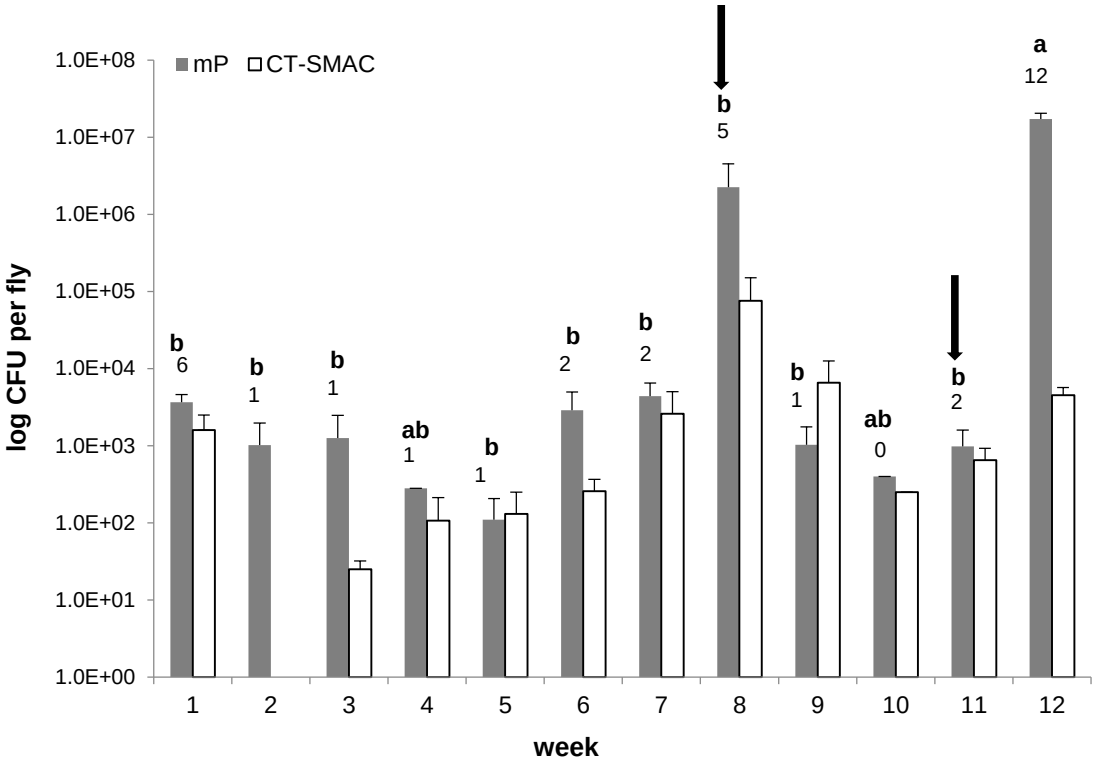

Figure 3.1 Prevalence and total CFU counts on mP agar and on CT-SMAC associated with stable flies on a beef cattle farm.

Numbers above the bars represent total number of flies processed each week by enrichment and IMS. Black down arrows above the bars indicate flies positive for *E. coli* O26 (in week 8) and O45 (in week 11). Different letters above grey bars indicate significant differences ($P < 0.05$) among CFU counts over 12 weeks.

The 12 week sampling period (June 9 - Aug 25) and a total of 15 stable flies were processed each week.

log CFU per fly is presented as mean±SEM. Detection limit for enterics was 10 CFU/fly on both, modified Posse agar (mP) and MacConkey agar with cefixime and tellurite (CT-SMAC).

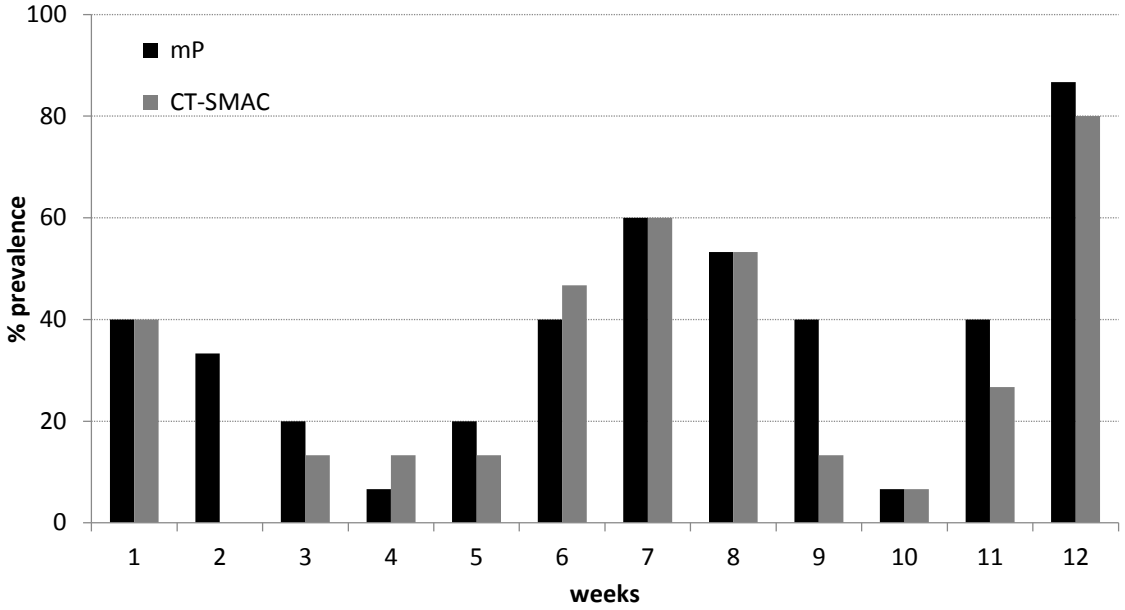

Figure 3.2 Prevalence of stable flies for CFU counts on mP agar and CT-SMAC by direct plating.

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Chapter 4 - Vector competence of house flies (*Musca domestica* L.) for seven serogroups of non-O157 Shiga-toxigenic *Escherichia coli* (STEC)

Introduction

House flies are synanthropic insects found all around the world. They are non-biting insects responsible for the transmission of foodborne pathogens. They transfer pathogens mechanically by means of body surface, by using a mode of feeding that involves regurgitation, and by fecal deposition (Graczyk et al. 2001). House flies collected from garbage dumps in Ethiopia were found to carry helminth and protozoan parasites. Both helminths (*Ascaris lumbricoides*, *Trichuris. trichiura*, hookworms, *Taenia* sp. and larvae of *Strongyloides stercoralis*) and protozoan species (*Entamoeba histolytica*/*dispar*, *Entamoeba coli*, *Giardia lamblia*, and *Cryptosporidium sp.*) were isolated from body surface and intestinal contents of the fly (Getachew et al. 2007). Pathogens *Ascaris lumbricoides*, *Entamoeba coli*, *Giardia lamblia* were also recovered from external body surface of house flies collected from restaurants, butcheries, and supermarkets in Iran (Motazedian et al. 2014).

Previous studies have shown that house flies are able to carry foodborne pathogens such as *Yersinia pseudotuberculosis* up to 36 h (Zurek et al. 2001), *Aeromonas caviae* up to 8 days (Nayduch et al. 2002), and *Staphylococcus aureus* up to 6 h (Nayduch et al. 2013) post infection in their digestive tract. In addition, Doud and Zurek (2012) showed that *Enterococcus faecalis* can multiply in the crop of house flies and bacteria persist in the midgut until 96 h post infection. Cefpodoxime resistant *Salmonella typhi* was recovered from the surface sterilized house flies in India (Vasan et al. 2008). The potential of house flies as a vector of dermatophyte *Microsporum canis* was studied by Cafarchia et al. (2009) and *M. canis* was observed until 4 h in the internal organs and on the outer body surface up to 5 days post infection.

Shiga-toxin producing *Escherichia coli* (STEC) are foodborne pathogens responsible for illnesses such as bloody diarrhea, hemorrhagic colitis, and life-threatening hemolytic uremic syndrome (HUS) (Tarr et al. 2005). STEC is estimated to cause more than 265,000 illnesses each year in the United States alone (Scallan et al. 2011). The major serogroup of STEC is O157; however, more than 200 serogroups of non-O157 STEC have been reported to cause STEC illness (Caprioli et al. 2005, Hedican et al. 2009, Käppeli et al. 2011). Both O157 and non-O157 STEC are associated with multiple outbreaks infecting thousands of people every year not only in the U.S. but globally, mainly affecting children of <4 years old (Majowicz et al. 2014). Infection is caused by Shiga-toxins belonging to two major gene families *stx1* and *stx2* along with intimin protein which is essential for the attachment to the intestinal epithelium and is encoded by *eae* (Gyles 2007). Treatment of STEC infection using antibiotics is controversial over long time (Carter et al. 1987, Dundas et al. 2001) and mainly includes supportive therapy. Human infections with non-O157 STEC has increased in recent years (Brooks et al. 2005, Johnson et al. 2006, Gould et al. 2013). Six serogroups of non-O157 STEC (O26, O45, O103, O111, O121, and O145) are responsible for more than 70% of non-O157 STEC infections to humans (Gloud et al. 2009, CDC 2011) and these serogroups are declared as adulterants in ground beef and non-intact beef products by the U.S. Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS). In addition, one rare serogroup of non-O157 STEC, O104 was responsible for a large outbreak in Germany in 2011 resulting in death of 39 people (Frank et al. 2011).

Escherichia coli O157:H7 were detected in house flies from cattle farm for the first time in Japan in 1999 (Iwasa et al. 1999) and were found to proliferate in the mouth parts of house fly causing bio-enhanced transmission of bacteria (Kobayashi et al. 1999). The frequent and high

($\sim 10^4$) bacterial excretion until 24 h indicates their potential to disseminate the pathogen through defecation, besides carrying *E. coli* O157 for 4 days in crop (Sasaki et al. 2000). *Escherichia coli* O157 was detected in spinach leaves exposed to house flies that ingested *E. coli* from inoculated manure and agar plates (Talley et al. 2009). Trans-stadial transmission of *E. coli* from larval to adult stage was also observed; however, the competition among the bacteria in the gut was excluded by inoculating flies only with *E. coli* (Rochon et al. 2004, 2005, Schuster et al. 2013). House flies were found to carry STEC O157 in cattle environment (Alam and Zurek 2004). They were also shown to transfer *E. coli* O157 to cattle, and to their drinking water (Ahmad et al. 2007). The objective of this study was to assess the vector competence of house flies for seven serogroups of non-O157 STEC (O26, O45, O103, O104, O111, O121, and O145) in laboratory bioassays mimicking the natural conditions.

Materials and Methods

House flies. House flies used in the experiment were from the laboratory colony at the Department of Entomology, Kansas State University. Flies were maintained at 70±10% humidity 25±2°C and an 18L: 6D cycle. They were provided with *ad libitum* water and sugar. Egg powder (Honeyville, Food Products, Honeyville, UT) was given to one week old flies as a source of protein for ovular maturation.

STEC-7. Seven serogroups of *E. coli*, JB1-95 (clinical isolate, serotype O111:H-), CDC 96-3285 (human stool, serotype O45:H2), CDC 90-3128 (human stool, serotypeO103:H2), CDC 97-3068 (human stool, serotype O121:H19), 83-75 (human stool, serotype O145:NM), H30 (infant with diarrhea, serotype O26:H11; Konowalchuk et al., 1977), ATCCBAA-2326 (human stool, serotype O104;H4; European outbreak) were used in the bioassays, provided by Dr. T. G. Nagaraja. These seven serogroups of *E. coli* have the resistance marker to rifampicin at the

concentration 100 mg/l. These were maintained on Tryptic Soy Agar (TSA) (Becton Dickinson, Sparks, MD).

Assay. Two-three days old mixed sex house flies were used from laboratory colony (n=60) for each bioassay. House flies were starved for 2 h before the assay. Flies were then exposed to freshly cultured inoculum of STEC (one serogroup) at a concentration of ~ 10^7 CFU/ml of phosphate-buffered saline (PBS) (pH 7.2; MP Biomedicals, Solon, OH). The concentration of inoculum was determined by spread plating on TSA and MacConkey agar (Becton Dickinson, Sparks, MD) and also on MacConkey agar supplemented with rifampicin (100 mg/l) (Sigma Aldrich, St. Louis, MO). Control flies were exposed to sterile PBS for 2 h.

Plastic petri dish 150 mm × 25 mm (Corning incorporated, Corning, NY) was used for the exposure and inoculum (~ 2 ml) was provided to flies in small petri dish 60 mm × 15 mm (Fisherbrand, Fisher Scientific LLC, Denver, CO) with sterile cotton to help flies land and feed (Figure 4.1). After the 2 h exposure to the individual serogroups of STEC, flies were randomly separated into groups of five per container in paper container 3 3/8" × 3 3/4" (Ridgid Paper Tube Corporation, Wayne, NJ) (Figure 4.2). Ten flies were surface sterilized using 0.5% sodium hypochlorite, 70% ethanol, and sterile water as described by Zurek et al. (2000) and homogenized individually in 1.0 ml PBS in 1.5 ml of microcentrifuge tubes (Fisherbrand, Fisher Scientific LLC, Denver, CO) using plastic pestles on each of 0, 1, 3, and 6 days post exposure. The homogenate was spread plated on MacConkey agar to monitor the background colonies and MacConkey agar with rifampicin for selecting STEC. Colony forming units (CFU) were counted after incubation at 37°C for 24 h. The control flies were processed on day 6 post exposure, same way as treated flies.

Results

Results from the bioassays revealed that house flies can carry STEC-7 for at least 6 days under laboratory conditions except for the serogroup O145. The abundance of bacteria declined sharply within the day 1 and then stabilized at ~ 2 log- 4 log CFU per fly for all the serogroups tested.

Serogroup O104: A 100% of flies were positive immediately after exposure (day 0) and the mean CFU count of bacteria per fly was $1.1 \pm 0.48\times10^5$. However, the prevalence of bacteria declined to 80% on day 1 and then increased to 90% on day 3 and 6 (Figure 4.3). The concentration of bacteria was also decreased gradually from day 1 to 6 except slight increase on day 3. The mean CFU counts of bacteria on day 1, 3, and 6 was $8.3 \pm 7.3\times10^2$, $2.0 \pm 1.9\times10^4$ and $1.2 \pm 0.6\times10^3$, respectively (Figure 4.3). The mean concentration of bacteria on water given to flies post exposure in paper can were $2.1 \pm 2.0\times10^3$, $1.3 \pm 0.0\times10^6$, and $2.1 \pm 0.01\times10^6$ CFU/ml on day 1, 3, and 6 respectively.

Serogroup O103: All the flies tested carried *E. coli* O103 on day 0 with the mean concentration of $3.1 \pm 1.0\times10^4$ CFU/fly. The prevalence of bacteria was reduced to 80% on day 1 and increased to 100% on day 3 and 90% on day 6. The mean CFU counts of bacteria on day 1, 3, and 6 were $1.3 \pm 0.6\times10^2$, $1.2 \pm 0.8\times10^3$, and $1.6 \pm 1.2\times10^2$, respectively. House flies processed on day 1 were both male and female and only males were processed in the experiment on rest of the days (1, 3 and 6) (Figure 4.4).

Serogroup O26: The prevalence of STEC was 100% on all days except day 1 (10%). The concentration of bacteria was $3.8 \pm 1.6\times10^3$ CFU/fly on day 0 and reduced greatly on day 1 with the mean count of $1.0 \pm 1.0\times10^1$ CFU/fly. The concentration of bacteria increased slightly

on day 3 (mean: $1.4 \pm 0.4 \times 10^3$ CFU/fly and decreased on day 6 (mean: $4.1 \pm 2.9 \times 10^2$ CFU/fly) (Figure 4.5).

Serogroup O121: This serogroup was 100% prevalent in house flies on all days 0, 1, 3, and 6 post inoculation; however, the concentration of bacteria reduced on day 1 and then stabilized until day 6 of post inoculation (Figure 4.6). The mean CFU counts were $8.9 \pm 2.1 \times 10^4$, $4.0 \pm 2.6 \times 10^3$, $9.0 \pm 5.1 \times 10^3$ and $5.3 \pm 1.2 \times 10^3$ CFU/fly on day 0, 1, 3, and 6, respectively. Of the entire house flies only males were processed on day 0, 3, and 6 days and mixed sex flies were processed on day 1.

Serogroup O45: All the house flies tested were positive on day 0 and day 3 (Figure 4.7). The prevalence of STEC O45 declined to 50% and 40% on day 1 and 6 respectively, post inoculation. The concentration of bacteria was high on day 1 with the mean count of $4.6 \pm 1.5 \times 10^4$ CFU/fly and decreased sharply on day 1, 3, and 6 and the mean CFU counts were $1.7 \pm 0.8 \times 10^2$, $3.5 \pm 1.3 \times 10^3$ and $2.2 \pm 2.1 \times 10^3$ respectively (Figure 4.7).

Serogroup O111: *Escherichia coli* were present in all the flies processed on day 0, 1, 3, and 6 and 60% of flies were positive on day 3 post inoculation (Figure 4.8). The concentrations of bacteria were also reduced by 1000 fold (from $1.0 \pm 0.2 \times 10^5$ to $4.3 \pm 2.7 \times 10^2$ CFU/fly) over first 24 h. The concentration of *E. coli* increased slightly on day 3 (mean: $2.2 \pm 0.8 \times 10^4$ CFU/fly) and decreased on day 6 (mean: $2.7 \pm 1.5 \times 10^2$ CFU/fly). All the house flies processed were male in the experiment.

Serogroup O145: The prevalence of STEC O145 was 100% on day 0 and declined gradually to 80% and 20% on day 1 and 3 respectively, post inoculation (Figure 4.9). The concentration of bacteria reduced over first 24 h but increased on day 3 and interestingly no flies were positive on day 6. The mean CFU counts of bacteria on MacConkey agar with rifampicin

was 1.2 ± 0.57×10^5, 1.4 ± 0.8×10^2 and 1.0 ± 0.9×10^4 on day 0, 1, and 3 post inoculation respectively.

Discussion

Vector competence of house flies for seven serogroups of non-O157 STEC was studied under laboratory conditions. Although several studies have addressed the association of STEC O157 and house flies, not much is known about non-O157 STEC and its ecology in relation to house flies. House flies have easy access to human refreshments and are a concern because of their synantropic nature and unrestricted movement. This is the first study investigating the association of house flies and non-O157 STEC.

Previously *E. coli* O157:H7 was investigated and detected up to 10^5 CFU/house fly on a cattle farm (Alam and Zurek 2004). We also found non-O157 STEC in house flies from feedlots (Chapter 2) indicating the role of house flies as vectors of non-O157 STEC in confined beef cattle environments. Also, Kobayashi et al. (1999) demonstrated that *E. coli* O157 can undergo multiplication in the house fly labellum. They exposed 6 to 8 days old female adult house flies to *E. coli* O157 with a concentration of ~ 10^9 CFU/ml for 30 minutes. Bacteria were detected until day 3 post inoculation in the digestive tract and in the excreta of house flies. Detection of bacteria for such a long period may be due to proliferation in the labellum. Similarly, GFP expressing *Enterococcus faecalis* OG1RF:pMV158 were shown to multiply in the crop of house flies (Doud and Zurek 2012). Two to five days old, mixed sex house flies were exposed to *E. faecalis* at a concentration of 3.1-7.8×10^6 CFU/inoculum (Doud and Zurek 2012). Highest bacterial concentration in the crop was observed 48 h post inoculation, and remained high until the end of experiment (96 h). The concentration of bacteria was observed lowest in the hind gut of flies throughout the bioassay period as bacteria were digested in the midgut. In our study, the

concentration of non-O157 STEC sharply declined within 24 h and then increased on day 3 post inoculations. This indicates that bacteria were first digested and then started multiplying in the intestinal tract of flies. Alternatively, cross contamination could have taken place between the flies, sugar and water after post inoculation in the paper can. Non-O157 STEC was detected in the water given to the flies throughout the sampling period (1, 3, and 6 days post exposure). Flies might have contaminated water and sugar cubes during feeding and regurgitation. The 1000 fold increase in the concentration of *E. coli* between days 1 to 3 in water may be due to the multiplication of bacteria in the presence of moisture, sugar, and egg powder. It could also be possible that bacteria multiplied in the fly labellum or/and crop then deposited in water during each feeding. Nonetheless, we tried to mimic the natural conditions where flies could be exposed to contaminated food or manure repeatedly. The results of this experiment show that seven serogroups of non-O157 STEC can persist in the house fly gut for at least 6 days (except for O145) if food/water are available *ad libitum*.

The upregulation of antimicrobial peptides (AMPs) and lysozyme gene expression was detected in the head and salivary gland of house flies fed GFP expressing *E. coli* O157:H7, whereas minimal upregulation was observed in the gut (Fleming et al. 2014). Steady decrease in the bacterial load was observed up to 12 h which was due to immobilization within the peritrophic membrane, lysis and peristaltic excretion. However, intact bacteria were observed in the crop and rectum (Fleming et al. 2014), suggesting the role of house fly as a vector of this pathogen for at least 12 h. Ahmad et al. (2007) observed *E. coli* O157 up to the concentration 1.1×10^{6} CFU/g of cattle feces post exposure to house flies inoculated with nalidixic acid resistant *E. coli* O157 strains in the concentration 1.2×10^{8} CFU/ml for 48 h. *Escherichia coli* were observed until day 11 in feces of all calves. Bacteria were also observed sporadically in water

given to the calves. Non-O157 STEC in our study were recovered from water given to flies indicated that flies also transmit non-O157 STEC to their environment. Sasaki et al. (2000) detected *E. coli* O157 in the crop of house flies for at least 4 days post inoculation. The concentration of bacteria was 5×10^6 CFU/crop and was reduced to $10^3 – 10^4$ CFU/crop on day 4. This indicated that bacteria did not undergo multiplication in the crop and other parts of digestive tract in the house fly. The prevalence of *E.* coli O157 positive flies also declined to 25% on day 4 from 100% on day 0 (Sasaki et al. 2000). *Escherichia coli* O157 was also detected in the fly's excreta in 1 and 3 h post inoculation at the concentration of 10^4 and 1.8×10^4 CFU/excreted drop, respectively and decreased in 24 h. The percentage of positive drops also decreased in 24 h (Sasaki et al. 2000). The concentration and prevalence of *E. coli* O157 from these studies was similar to non-O157 STEC serogroups in our study (except the increase of the bacterial concentration on day 3). However, we did not monitor the bacterial population density in different parts of digestive tract (crop, midgut, and hindgut).

In another study, Kobayashi et al. (2002) investigated the potential spread of *E. coli* O157 through fly excreta. House flies were able to transfer bacteria to food (boiled potato and raw beef). The concentration of bacteria decreased over time and maximum numbers of *E. coli* were observed at 3 h post inoculation. House flies that ingested *E. coli* O157 were able to contaminate the food and when it was incubated at 29°C for 24 h, *E. coli* multiplied rapidly indicating the proliferation of bacteria in food in case of improper storage. Similar to this, flies might have contaminated non-O157 STEC in the food (sugar cube with egg powder) given to flies in bioassay through their excreta and therefore recontamination and multiplication over time might have taken place.

In the present study, we showed that non-O157 STEC are carried by house flies over a considerable period of time and they are, therefore, potential vectors of non-O157 STEC. We observed bacteria with a concentration 10^2 -10^4 CFU/fly on day 6 post inoculation for all serogroups tested except O145. It is possible that *E. coli* O145 was lysed in the gut of the house fly like other microbes such as *Aeromonas hydrophila* (McGaughley and Nayduch, 2009). It might also be more susceptible to the antimicrobial peptides of the house fly immune system and, therefore could not survive after day 3 in the gut of house flies. In contrast, *E. coli* O121 was present in all flies throughout the bioassay period. *Escherichia coli* O121 may be more resistant to antimicrobial peptides and lysozymes. These results show that non-O157 STEC serogroups are unique and may affect insect immune systems differently. Future research will focus on the transfer of non-O157 STEC from the gut of fly to the surrounding environment and human food. The next step of this research is to assess the persistence and competition of bacteria in gut of the house fly upon exposure to a mixture of STEC-7.

Conclusion

Seven serogroups of non-O157 STEC were detected until day 6 post inoculation for all the serogroups tested (except O145) which suggests that house flies can carry non-O157 STEC in their digestive tract for a considerable amount of time. In conclusion, the house fly may play an important role as a vector and reservoir of seven serogroups of non-O157 STEC.

Figures

Figure 4.1 Experimental set up for exposure of house flies (n=50) to non-O157 STEC inoculum in PBS.

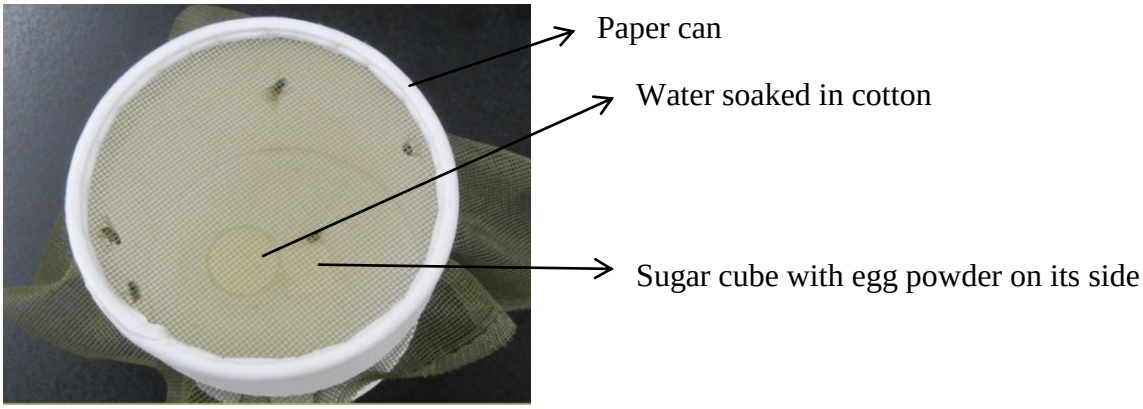

Figure 4.2 Rearing house flies (n=5) in paper can with sugar, water, and egg powder.

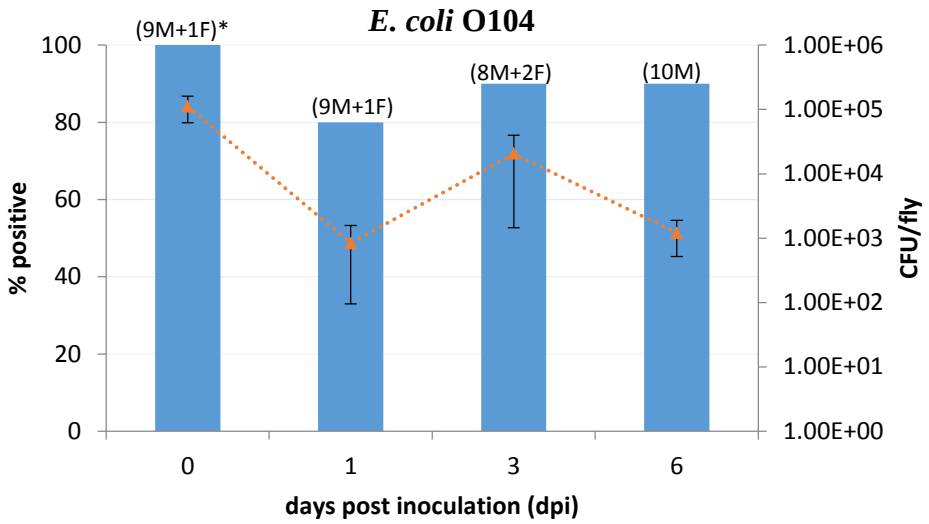

Figure 4.3 Bioassay with *E. coli* O104.

Error bars are standard of mean. *The number above the bar is number of male and female processed in the experiment (*M-male and F-female).

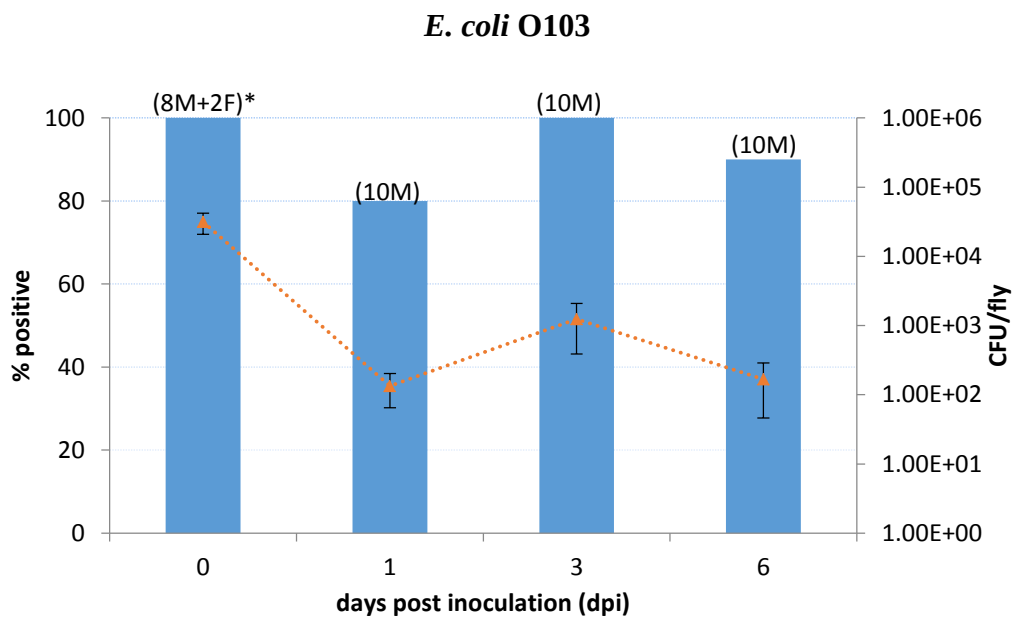

Figure 4.4 Bioassay with *E. coli* O103.

Error bars are standard of mean.*The number above the bar is number of male and female processed in the experiment (*M-male and F-female).

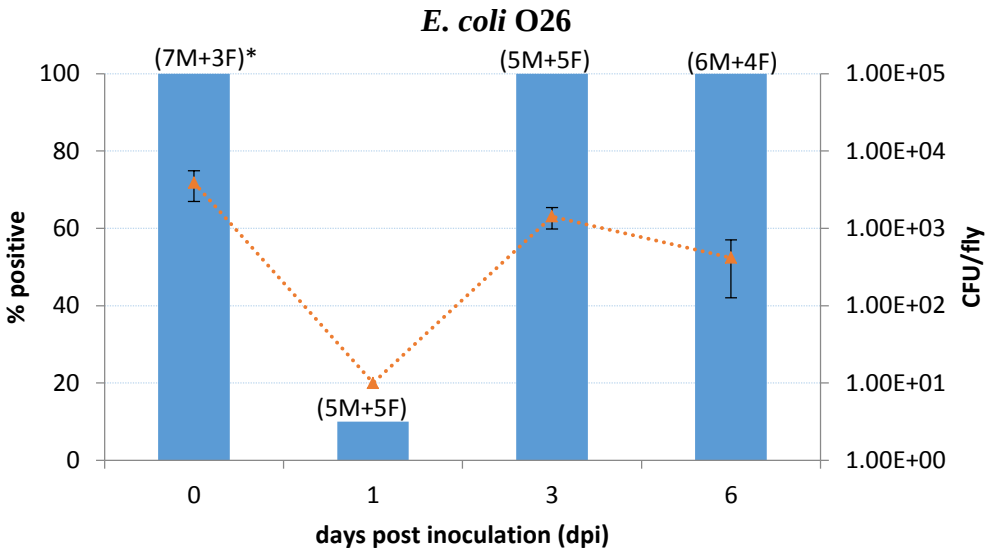

Figure 4.5 Bioassay with *E. coli* O26.

Error bars are standard of mean. *The number above the bar is number of male and female processed in the experiment (*M-male and F-female).

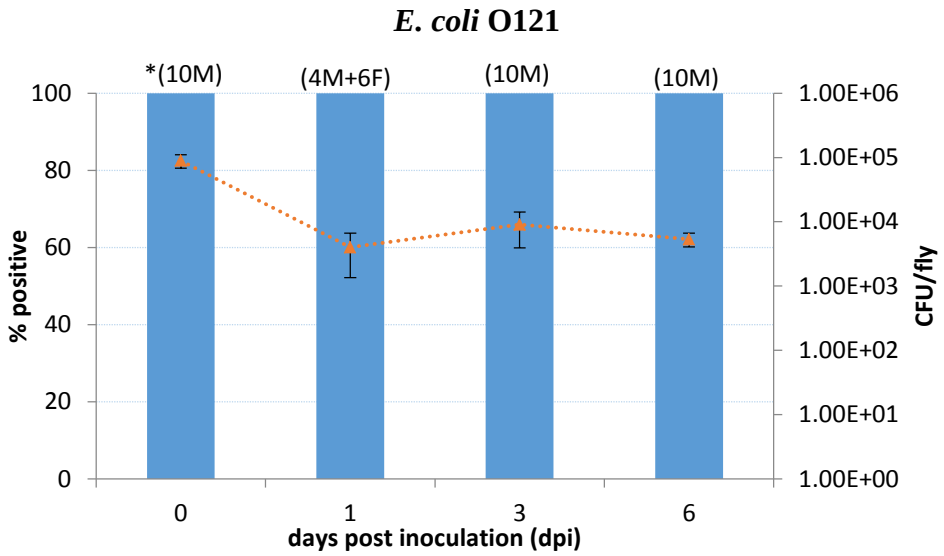

Figure 4.6 Bioassay with *E. coli* O121.

Error bars are standard of mean. *The number above the bar is number of male and female processed in the experiment (*M-male and F-female).

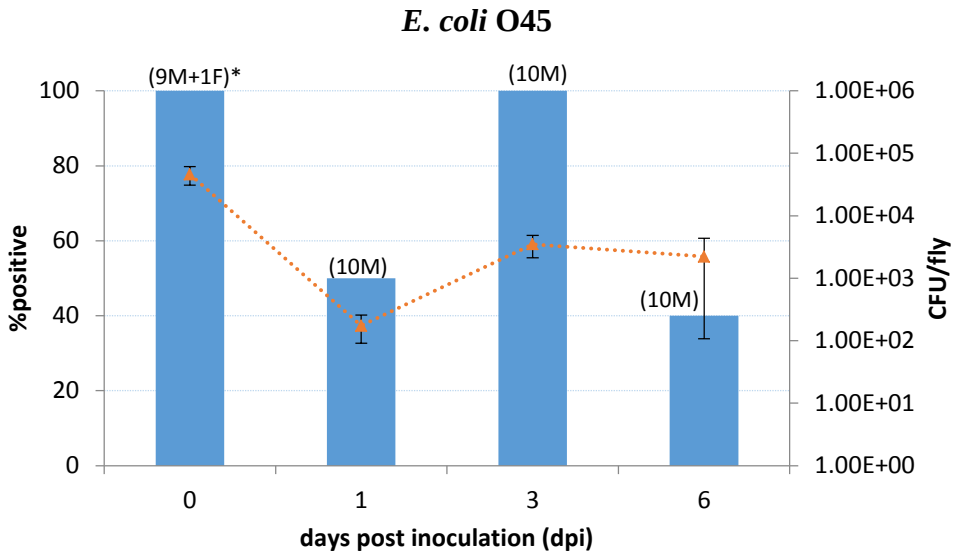

Figure 4.7 Bioassay with *E. coli* O45.

Error bars are standard of mean. *The number above the bar is number of male and female processed in the experiment (*M-male and F-female).

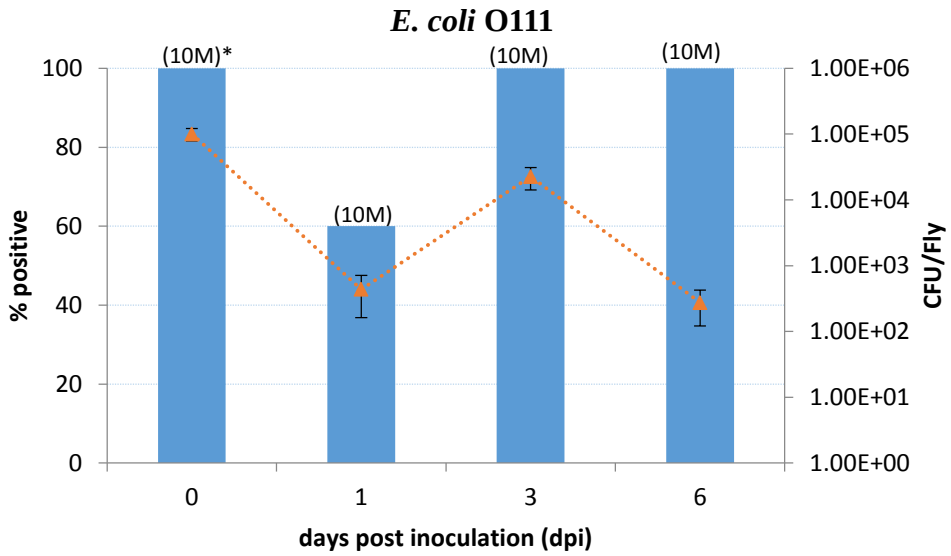

Figure 4.8 Bioassay with *E. coli* O111.

Error bars are standard of mean.*The number above the bar is number of male and female processed in the experiment (*M-male and F-female).

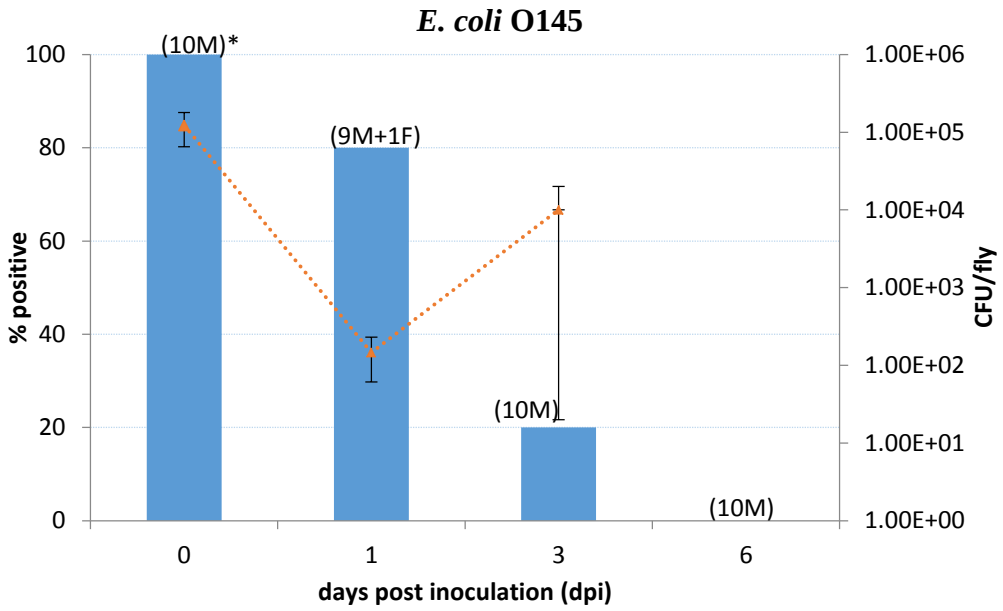

Figure 4.9 Bioassay with *E. coli* O145.

Error bars are standard of mean. *The number above the bar is number of male and female processed in the experiment (*M-male and F-female).

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Chapter 5 - Conclusion

House flies and stable flies, due to their larval developmental habitat may play an important role in the ecology and transmission of microorganisms that originate in cattle manure. Both muscoid fly species build up very large populations in confined cattle environments.

Shiga-toxigenic *Escherichia coli* (STEC) are important foodborne pathogens responsible for large human outbreaks in the United States and other parts of the world. There are about 30 cases of death every year in the United States due to STEC (CDC 2011). STEC O157 is the main cause of human illness; however, after 2005 infections with non-O157 STEC have increased (Scallan et al. 2011, CDC 2011). Six serogroups of non-O157 STEC are regarded as adulterants in beef products by United State Department of Agriculture Food Safety and Inspection Service (USFSIS). They are termed as "big six" serogroups which are responsible for more than 70% of non-O157 human illness. These include *E. coli* O26, O45, O103, O111, O121, and O145. In addition, there is another strain, *E. coli* O104, responsible for a large outbreak in Germany in 2011 killing 39 people (Frank et al. 2011). STEC causes bloody diarrhea, hemolytic uremic syndrome, hemorrhagic colitis, thrombocytopenia among others (Tarr et al. 2005, Johnson et al. 2006). Cattle are the asymptomatic reservoirs of STEC, and they shed bacteria intermittently in feces (Karmali et al. 2010). Human incidences of STEC are high during summer, which is also a peak season for flies, and flies get access to food easily during this season due to outdoor activities. However, not much is known about the ecology of non-O157 STEC and the role of insects in dissemination of these bacteria. The review of literature (Chapter 1) demonstrated that there is a gap in the information about the association of house flies with non-O157 STEC even though few studies have addressed the association of STEC O157 and house flies.

First, I assessed the prevalence of seven serogroups of non-O157 STEC (STEC-7) (O26, O45, O103, O104, O111, O121, and O145) in house flies from confined cattle environments. House flies were collected from the six feedlots and three dairy farms, and were subjected to the isolation of STEC by culture-based methods which included direct and enrichment plating on the selective differential medium, modified Posse (Possé et al. 2008). This was followed by multiplex PCR to differentiate serogroups and detect virulence traits. Although we observed a low frequency of STEC positive house flies (1.5% prevalence), this in livestock environment represents very large number of house flies and may pose a serious threat to cattle in addition to people working in beef and dairy cattle industry as well as the surrounding residential areas. I used similar protocols to assess the prevalence of STEC in stable files from a facility. STEC was not detected in stable flies even though bacteria were detected in the cattle manure in the same feedlot. Thus, there is likely no risk of stable flies to carry and disseminate STEC. Further research is needed to confirm these findings in other feedlots, dairy farms and animal production systems since this study included only one feedlot for stable flies in the central United States.

Furthermore, vector competence of house flies for non-O157 STEC was assessed in the laboratory bioassays. Results revealed that house flies can carry non-O157 STEC for considerable period of time (at least 6 days after exposure, except for O145) in their digestive tract. The results described in this thesis revealed that unlike stable flies, house flies play a role as a vector of non-O157 STEC.

To the best of my knowledge, this is the first study to report the non-O157 STEC in house flies. Additional research is essential to describe if house flies play a role in dissemination of non-O157 STEC pathogens to cattle. They are potential vector of STEC because of their unrestricted movement, high attraction to human food and drinks, and their ability to carry

pathogens of human and animal feces origin. Overall, this thesis contributes to the limited body of data, summarizes current knowledge, and furthers the knowledge regarding the association and the ecology of non-O157 STEC with muscoid flies in confined cattle environments.

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