Preventing the Zoonotic Transmission of Shiga toxin-producing *Escherichia coli* and Subsequent Development of Post-diarrheal Hemolytic Uremic Syndrome at Animal Contact Venues using a One Health Approach

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Dedication

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I miss you both very much.

Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are a group of enteric bacteria that can cause severe illness in people, particularly young children. STEC infection can progress to the development of post-diarrheal hemolytic uremic syndrome (HUS) which can lead to renal failure and even death in the most severe cases. Ruminant animals like cattle, sheep, and goats, are natural reservoirs of STEC and thus, interacting with these animals can pose a human health risk. The purpose of this dissertation is to consider STEC transmission and subsequent HUS development from animal contact venues as a system connected by elements of human health, animal health, and environmental health and propose ways to mitigate disease risk at these events simultaneously across all three domains.

In a series of four chapters, this thesis identifies a relationship between animal exposure and HUS development independent of known risk factors for HUS, analyzes the effect of ruminant exposure setting on HUS development, determines which public health interventions are most effective at maximizing handwashing behavior at agricultural fairs, and offers pilot data measuring serum cortisol levels in Minnesota show cattle along with matched cattle from farm locations that are not for show. The combined findings from these studies a) provide evidence of an otherwise unexplored relationship between ruminant exposure and HUS, b) showcase that all children, regardless of prior farm exposures, have an increased risk of HUS when visiting a farm or other animal contact venue, c) demonstrate the impact that various social influences can have on handwashing behavior and propose ways to improve handwashing at animal contact venues, and d) offer pilot data to inform future research on cattle stress at agricultural fairs and petting zoos.

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Chapter 1 Introduction

Background and History of Shiga toxin-producing Escherichia coli and Post-diarrheal Hemolytic Uremic Syndrome

Shiga toxin-producing *Escherichia coli* (STEC) are a pathogenic group of *E. coli* bacteria that are transmitted fecal-orally to humans [1]. STEC was first discovered in 1977, and in 1983, infection with STEC was linked to the development of diarrhea-associated hemolytic uremic syndrome (HUS), a disease characterized by a triad of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal injury [2-3]. Since these discoveries, post-diarrheal hemolytic uremic syndrome has been established as a major cause of acute renal failure in otherwise healthy American children [4].

STEC are gram negative bacilli, and there are seven major STEC serogroups found in North America that cause the majority of infection in humans. These are commonly referred to as the "top-7" and include O26, O45, O103, O111, O121, O145, and O157 [5-8]. STEC bacteria colonize in the intestinal epithelium through attaching and effacing lesions, and they release antigenically distinct AB_5 toxins (Stx1 and/or Stx2) which are internalized through receptor-mediated endocytosis [2,9]. Shiga toxins travel through the bloodstream and bind to receptors on endothelial and epithelial cells where they inhibit protein synthesis and cause cell damage and death[10-11]. These toxins have a particular affinity for cells of the central nervous system and kidney [5]. Shiga toxins are encoded by the genes *stx1* and *stx2* which have been acquired over time via transduction from lambdoid bacteriophages [3,9,12-13]. There are multiple variants (subtypes) of *stx* genes. There are four subtypes of *stx1*: *stx*1a, *stx*1c, *stx*1d, and *stx*1e and 12 subtypes of *stx2*: *stx*2a, *stx*2b, *stx*2c, *stx*2d, *stx*2e, *stx*2f., *stx*2g, *stx*2h, *stx*2i, *stx*2j, *stx*2k, and *stx*2l, however, this listing is subject to change given that researchers may identify new subtypes [14].

Symptoms of STEC infection range from non-bloody diarrhea to stools that are virtually all blood. HUS is the most severe sequelae of STEC infection. This occurs when Shiga toxins bind to renal glomerular endothelial cells [5,15-16]. Development of HUS has been primarily associated with *E. coli* serotype O157:H7, however, HUS development after infection with non-O157 STEC strains has been documented. The gene *stx2*, specifically *stx2a,* is most commonly associated with the development of HUS [17]. HUS develops in approximately 5-10% of people infected with STEC bacteria [18]. Rates of HUS have fluctuated over the last 20 years but have stayed approximately steady overall [19].

The virulence of STEC is influenced by factors attributed to the host, pathogen, and environment. Primarily, the production of Shiga toxins, specifically those encoded by *stx2* genes, and the production of intimin, encoded by the *eae* gene, is linked to increased virulence [20]. Intimin is a protein on the outer membrane of epithelial cells that mediates the attachment of Shiga toxin to enterocytes. However, it is noteworthy that in some cases, *eae*-negative strains were still associated with more severe clinical outcomes and some *eae*-positive strains have been found to be associated with more mild illness [21]. Young age (≤ five years) contributes to increased risk of HUS in humans [22]. About 15% of children under five years of age and 6% of people in all age groups who are diagnosed with serotype *E. coli* O157:H7 progress to HUS. Approximately 50% of children diagnosed with HUS require dialysis, and 3-5% of cases result in death [23].

According to national enteric disease surveillance, it is estimated that infection with STEC causes more than 265,000 illnesses, 3,600 hospitalizations, and 30 deaths per year in the United States [24]. STEC incidence nationwide has been steadily increasing over time, likely due to the increased use of culture-independent diagnostic testing (CIDT) in U.S. hospitals [25]. Data from 2009-2018 taken from the CDC National Outbreak Reporting System, where U.S. states self-report outbreaks of enteric illness, indicate that there have been 704 outbreaks of STEC resulting in 7,142 illnesses, 1,667 hospitalizations, and 39 deaths. The majority of outbreaks were foodborne (43%) followed by Indeterminate/Other/Unknown (23%), person-to-person (19%), animal contact (9%), waterborne (4%), and environmental contamination other than food/water (1%) [26].

The Zoonotic Transmission of Shiga toxin-producing Escherichia coli at Animal Contact Venues

In 1982, outbreak investigations of STEC associated HUS identified ground beef consumption as an epidemiologic link, which led to the establishment of ruminants as reservoirs of STEC [27]. These animals, which include cattle, sheep, and goats, are herbivores that ferment food using microbes in a specialized compartment called a rumen before digesting it [28]. Often ruminants are domesticated by humans and are commonly found on farms and at agricultural fairs. STEC can be transmitted to humans in water, food, soil, or surfaces contaminated with animal feces [29]. Several studies have linked areas with high cattle densities to increased STEC incidence in human populations [30-32].

Between 2009-2018, there have been 65 reported outbreaks of STEC associated with live animal contact in the United States, resulting in 618 illnesses, 125 hospitalizations, and 5 deaths [26].

Animal contact venues like farms, petting zoos, and agricultural fairs provide unique opportunities for zoonotic disease transmission due to the high contact rate between humans and domesticated livestock at these events. The transmission of STEC at animal contact venues is multifaceted and involves the combined forces of human behavior, animal health and wellness, and environmental contamination (**Figure 1-1**) [33].

Human Behavior

Handwashing with soap and water has been identified as the primary public health strategy to prevent human infection with STEC and other enteric illnesses [34]. A 2020 quantitative microbial risk assessment reported that washing hands using antimicrobial soap resulted in an approximately 40-fold reduction in the risk of *E. coli* infection compared to not washing hands [35]. Several outbreak investigations of STEC at animal contact venues have cited inadequate handwashing facilities as a contributing factor to disease incidence [36-40]. Given that fecal matter from animal fur or skin can easily be transmitted to humans through direct contact (e.g., petting, feeding), handwashing is essential to minimizing disease risk [41].

The consumption of food and beverages in animal areas has also been linked to the incidence of STEC and other enteric pathogens, indicating that separation of food and animal areas is integral to maintaining safe event spaces [42-46]. The cross-contamination of food with enteric bacteria can also occur if food workers handle live animals and subsequently handle food for human consumption [47-48]. In addition to food consumption, other hand-to-face activities can increase the risk of disease transmission in humans. Hand-to-face contact is more common in young children, who are at increased risk of severe infection [49]. It is also common for animal contact event attendees to bring inanimate objects into animal areas, for instance strollers, which can increase the risk of environmental contamination [50].

Animal Health and Wellness

The health and wellness of ruminant animals also plays a crucial role in mitigating disease risk at animal contact venues. STEC shedding in cattle is influenced by a variety of factors including lactation stage in dairy cattle, age, dietary changes, heat stress, and psychologic stress. Dairy cattle in their first lactation period have been found to be at a higher risk of STEC shedding when compared to cattle with more than one lactation period. Cattle in their first 30 days of lactation are also more likely to shed STEC compared to cattle that have been lactating for over 30 days [51]. Younger calves are more likely to shed higher volumes of STEC compared to adult cattle [52]. Shedding prevalence has been found to be highest in steer calves at the postweaning stage [53]. Cattle age also influences the distribution of STEC virulence factors and colonization factors [54]. Higher temperatures and associated heat stress have been linked to increased STEC shedding in cattle [51, 55-58]. Psychologic stress from transportation [59-60], pain (slaughter, castration, dehorning)

[61-63], feed withdrawal [64], loud and unfamiliar noises, restraint, contact with unfamiliar people and animals, or exposure to new and unfamiliar environments [65-67] can also lead to increased shedding. The stress hormones epinephrine and norepinephrine may also upregulate virulence mechanisms [68].

The commingling of animals, which is common practice at animal contact venues, increases the risk of pathogen transmission between animals [69]. This is especially true if super-shedding animals are present. It is estimated that 5-6% of cattle around the world naturally carry STEC O157:H7 [70]. However, there is a significant amount of variation in shedding between individual cows [71]. In cattle, it is known that cows identified as "super-shedders", or those that shed concentrations that are over 10^4 colony-forming units (cfu)/g feces, have a predominant role in the transmission of STEC O157:H7 in the environment [72]. Studies have observed that fecal samples from most STEC O157 positive cattle have fewer than 100 cfu/g feces. Levels of up to 10^7 cfu/g feces have been detected in a much smaller percentage of super-shedding cattle [73]. It is estimated that 20% of cattle that shed *E. coli* O157:H7 are responsible for 80% of disease transmission [74]. Sections of the bovine intestinal tract, including the colon, terminal rectum, and recto anal junction (RAJ) exhibit tissue tropisms that lead to localized colonization of STEC O157:H7 in those areas [75-76]. Studies have attributed colonization at the RAJ with super shedding in cattle [77]. Commingling may also introduce new strains into a single environment with the arrival of new ruminants [78]. Preventive measures, including frequently cleaning cattle feeders [51], altering the type of feed [79-85], avoiding fasting [86], probiotics [87-89], and vaccination [90] have been shown to be associated with reduced STEC shedding in cattle.

Environmental Contamination

Managing STEC shedding in ruminants is especially important because STEC can survive from months up to years in the environment [91-92]. An STEC outbreak investigation at an Ohio county fair, which included severe cases as evidenced by hospitalizations and HUS development, discovered that sawdust and other environmental samples were contaminated with STEC 42 weeks after the event [93]. Enteric outbreaks have been linked to environmental exposure after removal of animals [45], contact with manure [50,94], sitting on the floor of animal areas [50], and exposure to dust [93]. Fecal matter from ruminants can contaminate surrounding surfaces including animal bedding, railings, and other inanimate objects such as shoes, clothing, strollers, or toys[93, 95-96]. Fair settings also pose a unique risk because ruminant animal feces have the potential to contaminate water supplies. Several outbreaks have linked contaminated fair water, some of which was used for food preparation, to large-scale STEC outbreaks at animal contact venues [44, 97-98]. Events with animal contact also typically occur in warmer months where temperatures are higher which can stimulate the growth of bacteria in the environment [99-100]. These outbreak events exemplify the need for safe manure disposal and adequate water and sewage management at animal contact venues.

Although the relationship between STEC and ruminants has been well studied, the relationship between ruminant exposure and HUS development is not as well understood. Recent evidence has suggested a direct association between contact with live farm animals and progression to HUS among people with STEC, indicating that the source of exposure may have implications for virulence [101]. However, the mechanism behind this relationship is currently unknown and requires further study. In addition to environmental contamination concerns in the natural environment, it is unclear whether the environment or setting where a person is exposed to STEC also influences disease risk. Older evidence suggests that people who reside on farms and have repeated contact with ruminant animals (and thus, repeated exposure to STEC) have higher levels of antibodies to STEC. A case-control study in Ontario conducted from 1992-1993 compared STEC antibodies between 63 cases of *E. coli* O157:H7 from dairy farm families with 256 controls who resided in the urban center of Toronto. Lipopolysaccharide antibodies were three times as high in farm cases when compared to controls, and Stx 1 neutralizing antibody was 6 times as high in farm cases when compared to controls [102]. Another study conducted the same year tested both cattle and human fecal samples from southern Ontario counties for *E. coli* O157:H7, antibodies to Stx1, and O157 lipopolysaccharide. Of 355 people, 6.3% tested positive for *E. coli* O157:H7. Of 1458 cattle tested, *E. coli* O157:H7 was detected in 46% [103]. The immunity in humans that builds from exposure to farm animals and livestock through living or working on a farm may be protective against the development of HUS. However, this relationship has not been documented.

Applying a One Health Framework to Manage STEC Transmission and Subsequent Development of HUS at Animal Contact Venues

The transmission of STEC at animal contact venues closely and intricately connects the domains of human health, animal health, and environmental health (**Figure 1-1**). The One Health approach to research is an integrated and interdisciplinary framework that incorporates human, animal, and environmental health expertise to find holistic solutions to problems [104]. By studying STEC transmission using a One Health approach and simultaneously mitigating risk across all three domains, we can have more complete information about opportunities where we can intervene and prevent both outbreaks and sporadic infection in these kinds of settings with direct animal contact. To accomplish that, this work is divided into distinct chapters, each of which addresses a different component of STEC transmission and HUS development across a larger system of disease transmission (**Figure 1-1**).

Chapter 2, a preface chapter, establishes the relationship between exposure to farm animals and livestock and HUS development, independent of known risk factors for HUS, including age and Stx2 exposure. This was done using surveillance data from the Indiana Department of Health, retrieved from 2012-2018. Logistic regression and mediation analysis were performed to determine the direct effect of farm animal exposure on HUS and the percent of the effect mediated by exposure to *stx2* genes.

Chapter 3 further examines the relationship between ruminant exposure setting (ruminant exposure only from living or working on a farm, ruminant exposure only from visiting a farm or other animal contact venue, or ruminant exposure from both settings) and HUS development. Surveillance data from the Minnesota Department of Health from 2010-2019 were retrieved for this analysis. Logistic regression was performed to determine whether, among STEC confirmed cases, exposure setting was associated with HUS independent of age, detection of *stx2* genes, and county ruminant per capita.

Chapter 4 examines common interventions used at animal contact venues to promote handwashing, and determines which interventions are most effective at optimizing handwashing at these events. A discrete choice experiment was conducted by administering surveys to fairgoers at the 2021 Minnesota State Fair to design to examine the relative effects of four attributes (resource accessibility, time availability, consequences, and social influence) on handwashing probability.

Chapter 5 investigates the relationship between transportation and show activities and stress in Minnesota cattle. A prospective cohort study was conducted on eight pairs of cattle, where each pair consisted of a show animal and a matched animal that remained at the home farm location throughout show activities. Serum samples were collected at three time points throughout the showing process, and serum cortisol levels were compared between show and farm cattle.

Although the benefits of agricultural fairs, which offer economic opportunity for farm families and applied academic opportunities for youth in the United States, are evident, the interaction between people, farm animals, and the environment can pose a biosecurity risk. The combined results from all four studies provide substantive insights into the transmission dynamics of STEC at animal contact venues. Fair organizers and event planners will be able to use the results of this work to inform biosecurity interventions at their events.

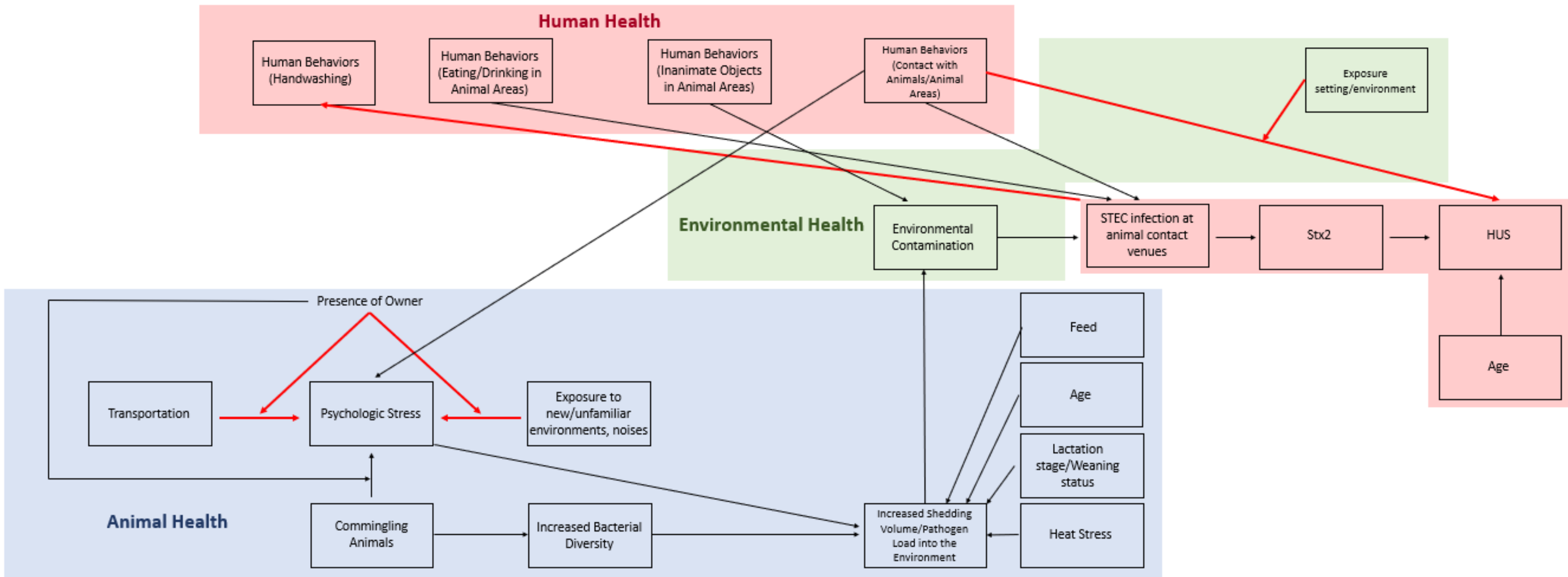

Figure 1-1 *Conceptual Model of Zoonotic Transmission of Shiga toxin-producing Escherichia coli and Diarrhea-associated Hemolytic Uremic Syndrome Development at Animal Contact Venues with Study Objectives Highlighted in Red*

Chapter 2 Preface: Farm Animal Contact is Associated with Progression to Hemolytic Uremic Syndrome in Patients with Shiga Toxin-Producing Escherichia coli — Indiana, 2012–2018

Vachon MS, Khalid M, Tarr GAM, Hedberg C, Brown JA. (2020). Farm animal contact is associated with progression to Hemolytic uremic syndrome in patients with Shiga toxin-producing *Escherichia coli* - Indiana, 2012-2018. *One Health*, *11*:100175. doi: 10.1016/j.onehlt.2020.100175. PMID: 33392374; PMCID: PMC7772627. (Original publication public domain)

Introduction

Hemolytic uremic syndrome (HUS) is a serious condition characterized by hemolytic anemia, thrombocytopenia, and acute renal dysfunction [105]. HUS can be precipitated by infection with several different bacterial enteric pathogens; the leading cause of post-diarrheal HUS in young children is Shiga toxin-producing *Escherichia coli* (STEC) [106]. *E. coli* O157:H7 is the STEC serotype most commonly associated with HUS. Approximately 15% of children younger than five years of age and 6% of people in all age groups who are diagnosed with *E. coli* O157:H7 progress to HUS. Dialysis is required in over 50% of children diagnosed with HUS and 3-5% of cases result in death [23]. Although infection with most non-O157 serotypes of STEC is less likely to result in severe clinical consequences, HUS has been observed in these cases [106].

STEC bacteria are transmitted fecal-orally [1]. Healthy ruminant animals, including cattle, goats, sheep, and deer, are natural reservoirs for STEC. Because ruminants do not have specific cell receptors that allow Stx to enter endothelial cells, they carry STEC without experiencing illness [107]. STEC can be transmitted to humans in water, food, soil, or surfaces that have been contaminated with animal feces [29]. Incidence of both O157 and non-O157 STEC have seasonal variability, with disease incidence peaking in the summer months [108].

Shiga toxin (Stx) is the principal virulence factor associated with the severe sequelae of STEC infections and is encoded by *stx1* and *stx2* genes [109]. Both Stx types have the same mode of action but are antigenically distinct [14]. Presence of *stx2* variants has been more commonly associated with HUS onset [110-111]. The high risk of HUS associated with Stx2 production is likely due to the toxin's greater ability to pass through epithelial cells of the intestine and enter the bloodstream, where it has toxic effects on the renal endothelium and blood cells [112].

The severity of clinical outcomes as a result of STEC infections has not previously been attributed to specific sources of exposure. An association between exposure source and virulence may allow clinicians to better predict the likelihood of progression to HUS. This information would also enable public health efforts to target exposure sources with the greatest impact on severe disease. This may be particularly relevant in states with greater animal agriculture operations. As of 2017, the state of Indiana was home to 56,649 farming operations. There were 17,014 farms with 844,187 cattle and calves. There were 11,753 farms with beef cows and 2,049 farms with dairy cows. Approximately 90% of farms with cattle had less than 100 animals per farm and 0.4% had 1,000 or more animals per farm [113]. STEC prevalence has been observed to be higher in beef cows than in dairy cows, and smaller farms have been associated with fecal shedding of Shiga toxin-encoding bacteria when compared to larger herds, likely due to variation in biosecurity practices [51, 114].

We conducted a population-based study in Indiana to examine the effects of direct contact with farm animals and livestock on HUS onset, overall and independent of potential mediating effects by *stx2* detection.

Methods

Laboratory-confirmed STEC cases reported to the Indiana State Health Department (ISDH) from 2012–2018 were retrieved for analysis. Indiana laboratories are required to forward positive STEC isolates to the ISDH laboratory for serotyping immediately upon identification, and hospitals are required to report confirmed cases of HUS to the ISDH immediately upon diagnosis [115]. Confirmed STEC cases were determined based on the national surveillance case definition applicable to the year of disease notification. Confirmatory laboratory evidence included either isolation of *E. coli* O157:H7 or isolation of other non-O157 strains supplemented by *stx* detection or evidence of Stx production [116-118]. Among the STEC cases, HUS cases were classified according to the national surveillance case definition, which requires acute illness diagnosed as HUS or thrombotic thrombocytopenic purpura (TTP) accompanied by anemia and renal injury [105]. Medical records were reviewed to verify clinical diagnoses of HUS for all HUS cases reported to ISDH.

All STEC patients were interviewed at the time of case reporting to determine potential sources of exposure. Patients were asked if they had direct contact with farm animals and livestock in the two weeks prior to illness onset, and if so, what type of animal.

We summarized cases by serotype and *stx2* detection and compared the seasonality of farm animal contact between patients who did and did not develop HUS. Using logistic regression, we estimated the effect of direct contact with farm animals and livestock on HUS onset, adjusted for age as a continuous variable. Regression coefficients were exponentiated to obtain odds ratios (ORs), and exact 95% confidence intervals (CIs) were calculated.

We used mediation analysis to measure the average direct effect of farm animal contact on HUS independent of potential mediation by *stx2* (**Figure 2-1**) [119]. The direct effect was calculated as the difference in the potential HUS outcome between those with and without animal exposure for a given *stx2* status, assuming no interaction between the exposure and mediator. The indirect, or mediated, effect was calculated as the difference in the potential HUS outcome between those with and without *stx2* for a given animal exposure status. The average direct and indirect effects were calculated by averaging across the direct and indirect effects for both levels of *stx2* and animal exposure status, respectively. We also calculated the average causal mediated effect and percent of the total effect mediated. CIs were estimated using 10,000 bootstrap replicates. Data were analyzed using the STATA/IC™ Software Suite version 16.0.

Results

A total of 784 confirmed STEC cases were reported during the study period. Of these, 46 (6%) developed HUS. There were 176 STEC patients aged ≤5 years. Of these, 26 (15%) developed HUS (**Table 2-1**).

The most commonly reported serotype was *E. coli* O157 (Table 2). Among all patients with confirmed *E. coli* O157 only, 41 (10%) developed HUS; among *E. coli* O157 patients who were children ≤5 years, 26 (23%) developed HUS. All HUS cases in patients ≤5 years were attributable to *E. coli* O157. By contrast, 1% of *E. coli* non-O157 patients developed HUS, and none were ≤5 years old. Of *E. coli* O157 strains, 92.6% expressed *stx2* (**Table 2-2**).

Among all STEC cases, information regarding exposure to farm animals or livestock was available for 600 (77%). The distributions of age, sex, race, serotype, and *stx2* detection were approximately equal in subjects with and without available animal exposure information (**See Supplementary Table 2-4**). Of those with available exposure information, 138 (23%) reported animal exposure, of whom 24 (17%) progressed to HUS. Of the 462 cases without direct animal contact, 22 (5%) developed HUS. Among children under the age of 5, 40% of those with reported farm animal contact developed HUS. Among children aged 6-10, 28% of those with reported farm animal contact developed HUS (**Figure 2-2**).

Incidence of STEC increased in summer months with a peak in July. STEC cases with reported farm animal contact also peaked in July. However, HUS was proportionally most common among STEC cases with farm animal contact in the fall months; at its maximum in September, 30% of STEC cases with farm animal contact progressed to HUS (**Figure 2-3**). The only months when the incidence of HUS among STEC cases without farm animal contact exceeded that among cases with farm animal contact were January-March, when very few cases reported animal contact.

Overall, farm animal contact was significantly associated with HUS onset after adjusting for age (OR 3.40; 95% CI 1.81, 6.40) (**Table 2-3**). Both direct (independent of *stx2*) and indirect (mediated by *stx2*) effects were >0 (**See Supplementary Table 2-5**). For the direct effect, we estimated the odds of HUS were 3.13 (95% CI 1.64, 5.98) times higher for cases with farm animal contact. In that model, the odds of HUS were 5.00 (95% CI 1.91, 13.11) times higher for cases in whom *stx2* was detected. The proportion of the total effect mediated by *stx2* was 12.2% (95% CI 7.03%, 24.2%) **(See Supplementary Table 2-5**).

Discussion

The odds of HUS were over three times higher among STEC cases with farm animal contact than those without (OR 3.40; 95% CI 1.81, 6.40). We found that only 12% of this association was mediated by *stx2*, yielding a direct effect of OR 3.13 among Indiana residents diagnosed with STEC. A greater proportion of younger children with farm animal contact developed HUS. HUS was proportionally most common among STEC cases with animal contact in the fall, but absolute numbers were highest in the summer months.

Farm animal contact is a well-known risk factor for STEC infection [29, 120-121]. Ours is the first study of which we are aware that demonstrates a higher risk for developing HUS after direct exposure to farm animals and livestock. Clinical measures such as hemoglobin, leukocyte count and creatinine have been used to predict HUS development and subsequent outcomes [122-123]. Given the results of our study, measures of exposure such as farm animal contact and seasonality may also be considered when determining the prognosis for STEC cases.

Additionally, this information could be provided to public health agencies when healthcare providers report HUS, expediting the process of identifying potential sources of exposure. Rapid notification of enteric disease outbreaks is essential for effective response, identification of the source of contamination, and prevention of further morbidities [124]. Between 2009 and 2017, there were 57 animal-associated outbreaks of STEC reported to the National Outbreak Reporting System, resulting in 563 illnesses, 113 hospitalizations, and 3 deaths, demonstrating the need for providers to promptly recognize and report cases of STEC and HUS suspected to be associated with animal contact [26]. Additional research is needed to compare clinical outcomes of animal-associated STEC outbreaks in the United States to outbreaks of STEC associated with other sources.

Our mediation analysis demonstrated that only a small portion of the association between animal contact and HUS is due to *stx2,* implying the importance of other mechanisms. One potential mechanism is the dose received via direct animal contact. The pathogenicity of STEC is determined by both host and virulence factors, and STEC has a relatively low infectious dose of 10-100 CFU [20,110]. In cattle, *E. coli* O157 typically adhere to and colonize the recto-anal junction of the gastrointestinal tract, which leads to pathogen shedding and subsequent contamination of the surrounding environment [76]. The term "super-shedder" is defined as shedding ≥ 10^4 CFU/g of feces; cattle that are super-shedders are capable of spreading significantly more pathogens when compared to other similar hosts [125-126]. Moreover, increased stress on animals exhibited in public settings, which may include transportation, confinement to limited physical space, overcrowding, over-handling, or comingling, can increase shedding and increase the risk of spreading harmful pathogens to people [33]. The association between animal contact in these settings and HUS should be studied. Several studies have been conducted to categorize STEC serotypes and virulence factors in ruminant animals, although few have compared the distribution of virulence factors in animals in public contact settings with free-range animals [127-129]. Analysis of feces collected from ruminant animals at agricultural events such as fairs, festivals, and petting zoos could help characterize both the pathogen load being shed into the surrounding environment and the distribution of known virulence factors in these settings, such as detection of stx_{2c} variants and activation of *eae* genes, that may inform pathogenicity of STEC strains [21, 130-131].

The proportion of HUS patients in our Indiana dataset diagnosed with *E. coli* O157 (10%) was on the higher limit of estimates reported in the literature (5–10%) [132]. We also observed a higher proportion of HUS cases among patients ≤5 years with confirmed *E. coli* O157 (23%) when compared with previous studies (14-15%) [23, 133]. This may be explained by our use of the CSTE case definition, which has been shown to overestimate the burden of post-diarrheal HUS [134].

In this study, animal exposure may have been misclassified. "Direct contact" may have been interpreted literally by respondents, even though contact with animal feces can occur without coming into physical contact with an animal. From a review of the comments sections in the surveillance data, some case investigators noted that patients who did not report direct animal contact did report consuming home-raised and home-butchered ground beef, visiting petting zoos, farms, or animal barns at state or local fairs, and residing near farms. This indicates that animal exposure may be underreported and/or underrecognized. Additionally, location of animal exposure was not documented in all case investigations during the study period precluding analysis of this characteristic. Location information would allow us to compare clinical outcomes among patients with exposures at agritourism events to patients who had contact with free range animals. Future studies may examine this.

Conclusions

Our results suggest that, among STEC cases, direct exposure to farm animals and livestock was a risk factor for HUS. Only a small portion of this association is mediated by *stx2*, suggesting the existence of other important mechanisms. This relationship between exposure source and severity of clinical outcomes has not been previously documented. Exposure variables, such as farm animal contact and seasonality, should be considered by clinicians when establishing the prognosis for STEC patients. Operators of venues allowing direct contact between farm animals and members of the public should take precautions to prevent transmission of STEC.

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Figures

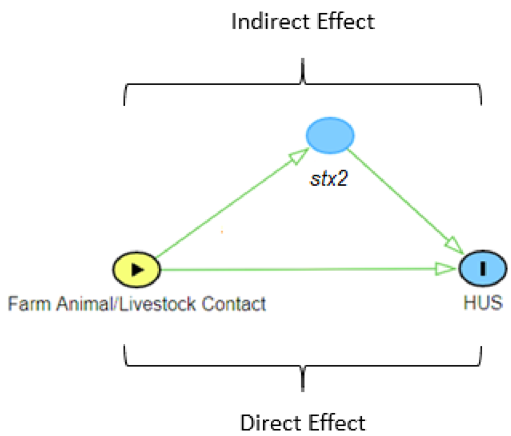

Figure 2-1. *Directed acyclic graph of hypothesized relationship between farm animal contact, age, stx2 genes, and HUS.*

The variable in yellow with the "►" symbol inside the oval is the exposure variable. The variable in blue with the letter "I" inside the oval is the outcome variable. Variables in blue are antecedents of the outcome variable. Abbreviations: HUS, Hemolytic Uremic Syndrome; stx2, Shiga toxin 2

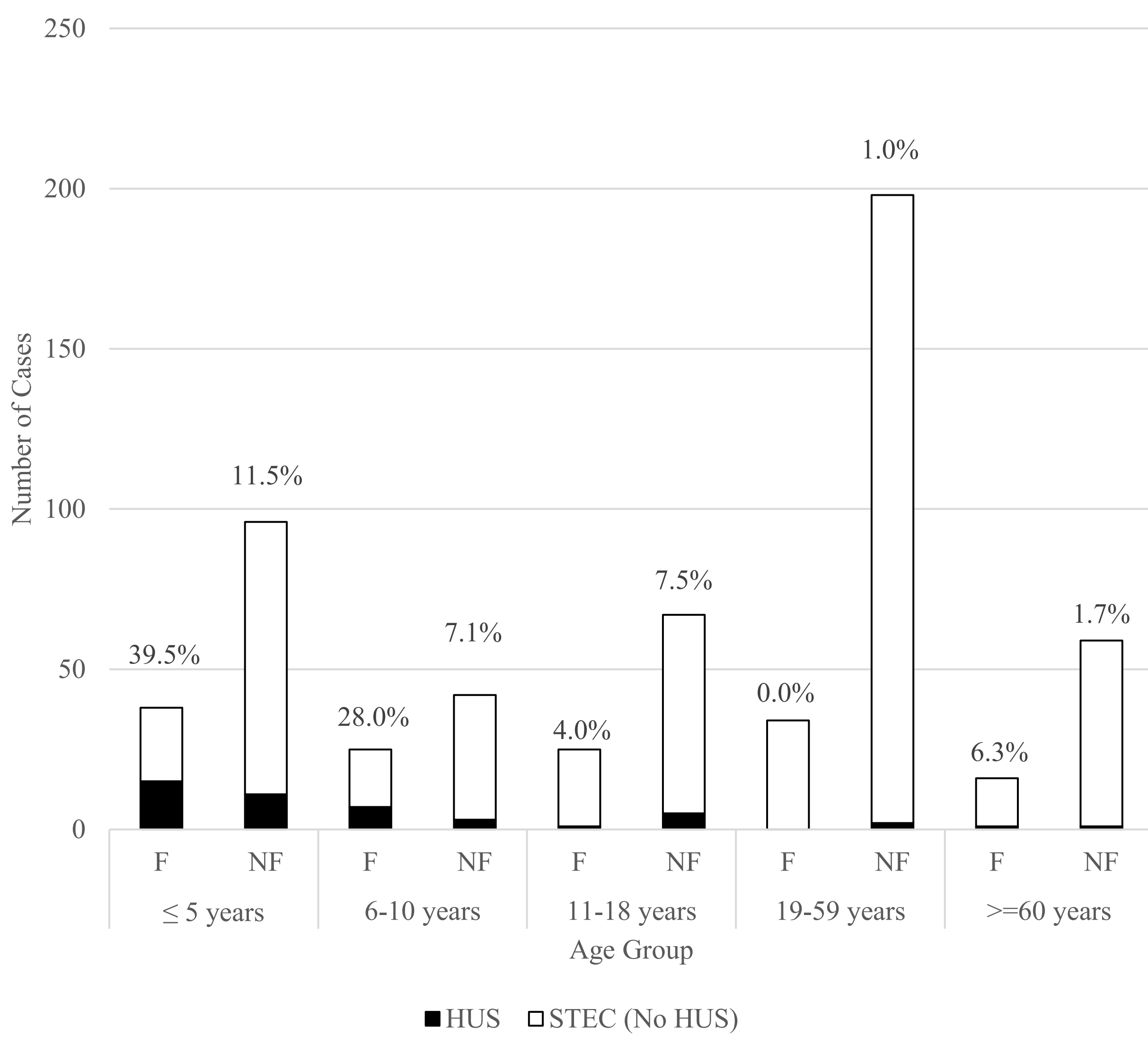

Figure 2-2. *Age Distribution of STEC and HUS by Known Farm Animal Contact and Percent HUS by Exposure Status.*

Patients with known farm animal exposure information were used to calculate the percent of patients who developed HUS by age by exposure status (n=600). The labeled percentages represent the percent of patients that developed HUS corresponding with the bar underneath. The majority of patients aged 5 years under and aged 6-10 years who developed HUS reported direct farm animal contact. Abbreviations: HUS, Hemolytic Uremic

Syndrome; STEC, Shiga toxin-producing *E. coli*; F, Farm Animal Contact; NF, No Farm Animal Contact

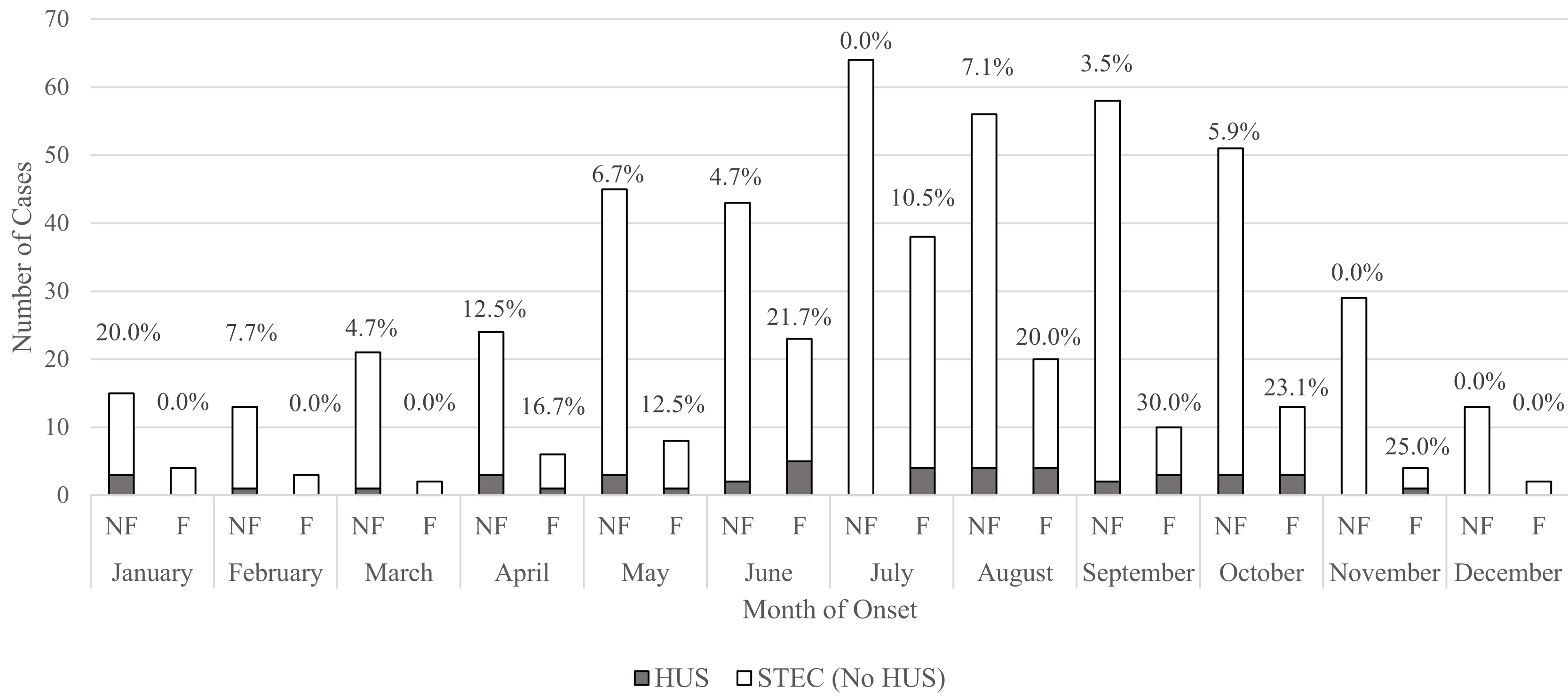

Figure 2-3. *Seasonal Distribution of STEC and HUS by Known Farm Animal Contact and Percent HUS by Exposure Status.* Patients with known farm animal exposure information were used to calculate the percent of patients who developed HUS by month by exposure status (n=600). The labeled percentages represent the percent of patients that developed HUS corresponding with the bar underneath. STEC incidence peaks in July independent of reported farm animal exposure. There is a greater proportion of HUS cases with farm animal contact between April and October, with the highest percentage of HUS cases with farm animal contact reported in September. Abbreviations: F, Farm Animal Contact; NF, No Farm Animal Contact

Tables

Table 2-1. *Demographic Distribution of Shiga Toxin-Producing Escherichia coli (STEC) cases, by Post-diarrheal Hemolytic Uremic Syndrome (HUS) Status — Indiana, 2012–2018.*

	STEC Patients			
Characteristic	**NO HUS**		**HUS**	
	n	**(%)**	**n**	**(%)**
Age group				
≤5 years	150	(20.3)	26	(56.5)
6-10 years	71	(9.6)	10	(21.7)
11-18 years	112	(15.2)	6	(13.0)
19-59 years	315	(42.7)	2	(4.3)
≥60 years	90	(12.2)	2	(4.3)
Female sex	422	(57.2)	25	(54.3)
Race				
White	477	(64.6)	39	(84.8)
Black or African-American	28	(3.8)	--	
Asian	15	(2.0)	1	(2.2)
Native American or Alaska Native	1	(0.14)	--	
Other	9	(1.2)	--	
Unknown	208	(28.2)	6	(13.0)

Abbreviations: STEC, Shiga toxin-producing *E.* coli; HUS, Hemolytic Uremic Syndrome.

Table 2-2. *STEC Serotype and Detection of stx2 genes among STEC cases, by HUS status — Indiana, 2012–2018.*

	STEC Patients							
	No HUS (*n*=738)				**HUS (*n*=46)**			
	Serotype n (%)		***stx2* detected n (%)**		**Serotype n (%)**		***stx2* detected n (%)**	
O157	363	(49.2)	336	(92.6)	41	(89.1)	38	(92.7)
O103	112	(15.2)	11	(9.8)	0	(--)	--	--
O26	92	(12.5)	8	(8.7)	1	(2.2)	--	--
O111	80	(10.8)	15	(18.8)	1	(2.2)	0	(0.0)
O45	32	(4.3)	5	(15.6)	0	(--)	--	--
O145	25	(3.4)	16	(64.0)	1	(2.2)	0	(0.0)
O121	18	(2.4)	15	(83.3)	2	(4.3)	2	(100)
O113	1	(0.1)	1	(100.0)	0	(0.0)	--	--
O28	1	(0.1)	1	(100.0)	0	(0.0)	--	--
O50	1	(0.1)	0	(0.0)	0	(0.0)	--	--
O69	1	(0.1)	0	(0.0)	0	(0.0)	--	--
O76	1	(0.1)	0	(0.0)	0	(0.0)	--	--
O8	1	(0.1)	1	(100.0)	0	(0.0)	--	--

O5	1	(0.1)	0	(0.0)	0	(0.0)	--	--
O103 & O111	2	(0.3)	1	(50.0)	0	(0.0)	--	--
O103 & O26	2	(0.3)	0	(0.0)	0	(0.0)	--	--
O103 & O121	1	(0.1)	0	(0.0)	0	(0.0)	--	--
O103 & O45	1	(0.1)	0	(0.0)	0	(0.0)	--	--
O45 & O26	1	(0.1)	1	(100.0)	0	(0.0)	--	--
O91 & O39	1	(0.1)	0	(0.0)	0	(0.0)	--	--
O118 & O111	1	(0.1)	1	(100.0)	0	(0.0)	--	--

Abbreviations: STEC, Shiga Toxin-Producing Escherichia coli; HUS, Hemolytic Uremic Syndrome; *stx2,* Shiga toxin 2 genes.

Table 2-3. *Association Between Farm Animal Contact and HUS adjusted for age (Total Effect) and independent of the mediating effects of stx2 (Direct Effect)— Indiana, 2012–2018.*

HUS	**Total Effect**		**Direct Effect**	
	OR	*95% CI*	*OR*	*95% CI*
Farm Animal Contact	3.40	(1.81, 6.40)	3.13	(1.64, 5.98)
stx2	--		5.00	(1.91, 13.11)
Age	0.94	(0.92, 0.97)	0.94	(0.92, 0.97)

Abbreviations: OR, Odds Ratio; CI, confidence interval; stx2, Shiga toxin 2.

Supplementary Tables

Table S-2-4. *Comparison of case characteristics between patients with and without animal exposure information available — Indiana, 2012–2018.*

Characteristic	Animal Exposure Information Available (n=600)	Animal Exposure Information Unavailable (n=183)
Age (years)—Median (IQR)	19 (6.0-38.0)	21(7.8-38.3)
Race, n (%)		
White	433 (72.2)	83 (45.4)
Black or African American	21 (3.5)	6 (3.3)
Asian	16 (2.7)	0 (0.0)
Native American or Alaska Native	0 (0.0)	1 (0.55)
Other	8 (1.3)	1 (0.55)
Unknown	121 (20.2)	93 (50.8)
Female sex, n (%)	351 (58.5)	96 (52.5)
Serotype, n(%)		
O157	314 (52.4)	90 (48.9)
O103	78 (13.0)	34 (18.5)

O26	78 (13.0)	15 (8.2)
O111	55 (9.2)	26 (14.1)
O45	24 (4.0)	8 (4.3)
O145	20 (3.3)	6 (3.3)
O121	19 (3.2)	1 (0.5)
O103 and O111	2 (0.3)	0 (0.0)
O111 and O118	1 (0.2)	0 (0.0)
O121 and O103	1 (0.2)	0 (0.0)
O26 and O103	1 (0.2)	0 (0.0)
O28	1 (0.2)	0 (0.0)
O45 and O26	1 (0.2)	0 (0.0)
O50	1 (0.2)	0 (0.0)
O69	1 (0.2)	0 (0.0)
O76	1 (0.2)	0 (0.0)
O8	1 (0.2)	0 (0.0)
O103 and O26	0 (0.0)	1 (0.5)
O113	0 (0.0)	1 (0.5)
O5	0 (0.0)	1 (0.5)
O91	0 (0.0)	1 (0.5)
stx2 expression n(%)	357 (60.7%)	96 (53.6%)
HUS	46 (7.7%)	0

Abbreviations: stx2, Shiga toxin 2; HUS, Hemolytic Uremic Syndrome.

Table S-2-5. *Mediation Analysis*

Effect	Mean	95% Confidence Interval	
ACME1	0.017	0.002	0.035
ACME0	0.007	0.001	0.016
Direct Effect 1	0.095	0.041	0.166
Direct Effect 0	0.085	0.037	0.152
Total Effect	0.102	0.050	0.172
% of Total via ACME1	0.170	0.098	0.338
% of Total via ACME0	0.074	0.043	0.147
Average Mediation	0.012	0.001	0.025
Average Direct Effect	0.090	0.039	0.159
% of Total Effect Mediated	0.122	0.070	0.242

The analysis assumed no interaction between the exposure and mediator. Abbreviations: ACME, Average Causal Mediation Effect.

Chapter 3 Farm Animal Exposure Setting Impacts Hemolytic Uremic Syndrome Risk Among Cases Infected with Shiga toxin-producing *Escherichia coli*— Minnesota, 2010-2019

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) transmission can occur at animal contact venues, which include agricultural fairs, petting zoos, and farm tours [33]. Ruminant animals, including cattle, sheep, and goats, are natural reservoirs of STEC [135]. Direct and indirect contact with these ruminants can increase the risk of STEC infection in humans [136-137]. During 2009-2018, there were 64 reported STEC outbreaks associated with animal contact in the United States, resulting in 618 illnesses and 125 hospitalizations [26]. Infection with STEC can lead to the development of post-diarrheal hemolytic uremic syndrome (HUS), which is characterized by a triad of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal injury. Progression to HUS is especially evident in younger age groups and among cases exposed to Shiga toxin 2 (Stx2) [15].

A previous study identified an association between farm animal contact and progression to HUS among STEC cases in Indiana. This association, which was independent of known risk factors for HUS (e.g., age, infection with an STEC strain that possesses *stx2*), indicates that the source of exposure could have implications for virulence [101]. Although earlier studies suggest that routine exposure to domesticated animals through living or working on a farm confers acquired immunity to STEC and its associated toxins, it is unknown whether HUS risk among STEC cases varies by the extent of prior exposure to farm animals [102-103].

In this study, we aimed to determine, using surveillance data from the Minnesota Department of Health (MDH), whether ruminant exposure setting influences HUS risk. We demonstrated that exposure to ruminant animals while visiting a farm or animal contact venue is an important risk factor for progression of STEC infection to HUS. Members of the public should adopt preventive public health practices (e.g., practice appropriate hand hygiene, avoid eating and drinking in animal areas, avoid bringing inanimate objects like strollers into animal areas) and take additional care when visiting animal venues with ruminants.

Methods

Data Collection and Inclusion Criteria

Laboratory-confirmed STEC cases reported to MDH from 2010 to 2019 were reviewed for analysis. Enteric *E. coli* infection is required to be reported to MDH, and a clinical specimen or bacterial isolate must be submitted to the MDH Public Health Laboratory [138]. STEC cases were deemed confirmed based on the Council of State and Territorial Epidemiologists case definitions associated with the year of disease notification. Evidence of confirmation included either isolation of *E. coli* O157:H7 or of non-O157 strains accompanied by either *stx* gene detection or evidence of Shiga toxin production [139]. Among cases with confirmed STEC, HUS case classification was in accordance with the national surveillance case definition, which mandates acute illness diagnosed as HUS or thrombotic thrombocytopenic purpura (TTP) accompanied by anemia and renal injury [140]. HUS is reportable to MDH immediately upon diagnosis [141]. Cases included in this analysis were those that tested positive for either *stx1* and *stx2* bacterial genes or *stx2* only, given that HUS is primarily associated with Stx2-producing strains [142].

As part of routine surveillance activities, all STEC cases were interviewed with a standard case investigation questionnaire. Cases were asked whether they lived on, worked on, or visited a farm in the 7 days prior to illness onset, or visited a petting zoo, educational exhibit, fair, or other venue with animals in the week prior to illness. Those responding 'yes' to any of the above were asked about contact with specific animals (e.g., cattle, goats, sheep), including an 'other' category.

STATISTICAL ANALYSIS

The primary outcome of interest was HUS development, a binary categorical variable. Because HUS risk among people who lived, worked, or visited a farm without ruminants (3.3%) was similar to HUS risk among people who did not live, work or visit a farm (4.1%), we classified our primary exposure variable as follows: 1) cases without any ruminant animal exposure; 2) cases whose only exposure to ruminants was because they lived or worked on a farm with ruminants; 3) cases whose only exposure to ruminants was because they visited a farm or animal contact venue with ruminants; and 4) cases who had exposure to ruminants because they both lived or worked on a farm with ruminants AND visited a farm or animal contact venue with ruminants. Ruminant exposure was defined as direct contact with a ruminant or contact with a ruminant animal's environment.

A descriptive analysis of the data was performed to determine the distribution of cases by STEC serogroup, detection of *stx* genes, age group, and exposure setting. We also examined the distribution of ruminants per capita in each county [30-32, 143-144]. Ruminants per capita were generated using cattle, sheep, and goat inventory from the United States Department of Agriculture (USDA) 2017 Census of Agriculture and population estimates from the Minnesota State Demographic Center [145-146]. For continuous outcomes, bivariate comparisons were made using a two sample t-test for binary predictors and one-way analysis of variance (ANOVA) for categorical predictors with three or more categories. For binary outcomes, bivariate comparisons were made using a chi-squared test for binary categorical predictors.

We performed multiple imputation by chained equations to handle missing data using the R package "mice" (Supp) [147]. We confirmed the relationship between any ruminant exposure and progression to HUS by fitting a logistic regression on each of the imputed datasets and adjusting for age and *stx* profile (Supp.). For our primary analysis, we fit a logistic regression on each of the imputed datasets with HUS development as the dependent variable and exposure setting as independent variable adjusted for age, *stx* profile of the STEC strain, and county ruminant per capita. We examined the interaction between age and exposure setting and used a likelihood ratio test to assess the change in residual deviance between the full and reduced model. The interaction term was dropped from our final model after it was determined that the difference between the two models was not significant. Regression coefficients were exponentiated to obtain odds ratios (ORs), and 95% confidence intervals (CIs) were calculated from pooled standard errors obtained using Rubin's rules [148].

Results

During 2010-2019 in Minnesota, there were 1,660 STEC confirmed cases with strains that tested positive for either *stx1* and *stx2* or *stx2* only. Of these, 377 (23%) were aged 5 years or under. The majority of cases (1147; 69%) tested positive for STEC O157. In total, 103 cases (6%) developed HUS. Of children aged 5 years or under, 58 (15%) developed HUS (**Table 3-1**). There was a significant difference in mean county ruminant per capita by exposure setting (F=9.96, p<0.0001). Mean county ruminant per capita was significantly higher in counties where cases whose only ruminant exposure was from living or working on a farm compared to cases with no ruminant exposure (p<0.0001). There was a significant association between cases who tested positive for *stx2* only and HUS development compared to cases who tested positive for both *stx1* and *stx2* (Chi-square = 18.2, p<0.0001).

In our sample, 1,350 cases (81%) did not report any ruminant exposure, 88 (5%) only had exposure to ruminants because they lived or worked on a farm with ruminants, 194 (12%) only had exposure to ruminants because they visited a farm or other animal venue with ruminants, and 28 (1.7%) both lived or worked on a farm with ruminants AND visited a farm or other animal venue with ruminants (**Table 3-2**). In our final adjusted model, ruminant exposure only from living or working on a farm was not significantly associated with HUS compared to STEC cases without any ruminant contact or exposure (OR: 1.29; 95% CI: 0.53, 3.12). Conversely, having ruminant exposure only from visiting a farm or other venue was associated with HUS (OR: 2.53; 95% CI: 1.51, 4.25). Ruminant exposure from both visiting a farm or other animal venue AND living or working on a farm was also associated with HUS after adjusting for covariates of interest (OR: 4.03; 95% CI: 1.52, 10.64) (**Table 3-3**).

Discussion

Our findings confirm that ruminant animal exposure increases the risk of HUS development among people with STEC infection. Specifically, HUS risk significantly increased among people who were exposed to ruminants while visiting a farm or other animal venue, with the magnitude of the risk differing slightly based on whether they also had contact with ruminants at home or work.

While several studies have established an increased risk of STEC infection due to direct ruminant contact [44, 149], living in a ruminant-dense area [30-32, 143-144], and visiting farms or petting zoos [39,50, 150], whether ruminant exposure is also associated with increased risk of HUS among individuals with STEC infections is less clear. More recent evidence indicated that the HUS rate from animal contact outbreaks (9%) was significantly higher than the HUS rate from outbreaks with other modes of transmission (6%) [151]. Our findings support these observations and suggest that animal contact venues may result in higher exposure doses or that strains circulating at these events may have increased virulence compared to strains encountered through other sources of exposure. Although county ruminant per capita has a large effect on infection risk, it had no effect on our estimates of HUS risk from animal exposure. This could be a consequence of either specifically examining HUS risk or from accounting for direct exposure in our model.

There are several potential explanations for why exposure to ruminants is associated with increased risk of progression to HUS among confirmed STEC cases. Stress associated with transportation and unfamiliar surroundings may cause ruminant animals to shed higher bacterial volumes at animal contact venues [152]. This would impact the exposure dose at such events. The commingling of a variety of animals also increases the diversity of bacterial strains contained in a single location [78, 153]. STEC isolated from ruminants harbor known virulence markers that contribute to clinical severity [20].

Although there has been evidence of acquired immunity to STEC and its associated toxins among adults who live or work on farms [102-103], our findings suggest that acquired immunity to home farm-specific strains may not be protective against other strains that may be present at animal contact venues, particularly among young children. We support this by showing that exposure to ruminants from both living or working on a farm AND visiting a farm or other public animal contact venue was associated with an increased HUS risk, with a higher odds ratio than that observed with visiting a farm or public animal contact venue only. However, all HUS cases in both categories were aged 10 or younger. Finally, behavioral changes may occur within the context of visiting a farm, agricultural fair, or petting zoo that may be less probable at a home farm location. Such behaviors may include eating or drinking in animal areas, increased contact rate between young children and high-risk animals, or bringing strollers, toys, and other inanimate objects into animal areas [42-46, 49-50].

The results of this study have implications for individual prevention, clinical awareness, and public health intervention. Parents of young children should remain cautious in all exposure settings with live ruminant animals given that immune mechanisms from routine exposure to these animals may not protect against severe clinical outcomes from STEC. Similarly, immune system decline in older adults may have contributed to the increased risk of HUS among STEC cases over the age of 65 years. Health care providers treating young children or older adults for acute STEC infections should be aware of the increased risk of HUS among cases who visited an animal contact venue with ruminants. Venue operators should make the public aware that exposure to farm animals and livestock from animal contact venues may place one at an increased risk of severe clinical consequences from infection, regardless of prior exposure or experience with animals.

This study was limited to STEC infections identified through pathogen-specific surveillance. Surveillance limitations, such as care-seeking biases, may impact the generalizability of our results. Inadequate sample size prevented us from examining non-linear relationships between age and HUS risk. We were also unable to examine potential mediation by serogroup or known virulence factors.

In addition to being a risk factor for STEC infection, exposure to ruminant animals may be an important predictor of HUS. Visiting a farm or other animal venue with ruminant animals may increase the likelihood of high risk STEC exposure. All members of the public should take additional care at public animal contact venues to avoid infection from animal contact. This can be done by practicing more frequent handwashing, avoiding food consumption or other hand-to-mouth contact in animal areas, and limiting strollers and other inanimate objects in animal areas.

Tables

Table 3-1. *Descriptive Summary of Laboratory-Confirmed Shiga Toxin-Producing Escherichia coli Cases by Exposure Setting, Age Group, Serogroup, Shiga Toxin Gene (stx) Profile, County Ruminant per Capita, and Hemolytic Uremic Syndrome (HUS) Status—Minnesota 2010-20*

	Cases without any ruminant animal exposure				Cases whose only exposure to ruminants was because they lived or worked on a farm with ruminants				Cases who had exposure to ruminants because they both lived or worked on a farm with ruminants AND visited a farm or other animal venue with ruminants				Cases whose only exposure to ruminants was because they visited a farm or other animal venue with ruminants.			
Age Group	**n**	**%**	**HUS**	**%HUS**	**n**	**%**	**HUS**	**% HUS**	**n**	**%**	**HUS**	**% HUS**	**n**	**%**	**HUS**	**%**
<=5 years	280	20.7[1]	35	12.5[2]	24	27.3	4	16.7	10	35.7	5	50.0	63	32.5	14	22.2
6-10 years	104	7.7	11	10.6	5	5.7	1	20.0	4	14.3	1	25.0	31	16.0	5	16.1
11-18 years	189	14.0	4	2.1	13	14.8	0	0.0	9	32.1	0	0.0	36	18.6	2	5.6
19-45 years	406	30.1	5	1.2	18	20.5	0	0.0	2	7.1	0	0.0	49	25.3	2	4.1
46-65 years	198	14.7	3	1.5	23	26.1	1	4.3	1	3.6	0	0	6	3.1	0	0.0
65+ years	173	12.8	9	5.2	5	5.7	0	0.0	2	7.1	0	0	9	4.6	1	11.1
Serogroup																
O157	928	77.5	63	6.8	59	71.1	5	8.5	21	77.8	3	14.3	139	77.2	20	14.4
O103	19	1.6	0	0.0	1	1.2	0	0.0	0	0.0	0	--	3	1.7	0	0.0
O26	26	2.2	0	0.0	1	1.2	0	0.0	0	0.0	0	--	2	1.1	0	0.0
O111	67	5.6	2	3.0	7	8.4	0	0.0	1	3.7	0	0.0	17	9.4	3	17.6
O145	60	5.0	0	0.0	4	4.8	0	0.0	4	14.8	2	50.0	8	4.4	1	12.5
O121	66	5.5	0	0.0	8	9.6	0	0.0	0	0.0	0	--	7	3.9	0	0.0

[1] Column percentage taken to determine case distribution by age group

[2] Row percentage taken to determine %HUS by age group

O45	5	0.4	0	0.0	0	0.0	0	--	0	0.0	0	--	0	0.0	0	--
Other	27	2.3	1	3.7	3	3.6	0	0.0	1	3.7	0	0.0	4	2.2	0	0.0
***stx* Profile**																
stx1 & *stx2*	608	45.0	17	2.8	35	39.8	2	5.7	11	39.3	0	0.0	118	60.8	8	6.8
stx2	742	55.0	50	6.7	53	60.2	4	7.5	17	60.7	6	35.3	76	39.2	16	21.1
County Ruminant per Capita	Med.	IQR	Med.	IQR	Med.	IQR	Med.	IQR	Med.	IQR	Med.	IQR	Med.	IQR	Med.	IQR
	0.21	0.86	0.24	1.17	1.3=3	1.69	1.05	0.66	0.80	1.22	0.25	0.92	0.38	1.22	0.27	0.93

Abbreviations: HUS, Hemolytic Uremic Syndrome; *stx*, Shiga toxin bacterial gene; Med., Median

Table 3-2. *Summary of Cases by Exposure Setting and HUS Status among Persons with Laboratory-Confirmed Shiga Toxin-Producing Escherichia coli Infections,—Minnesota 2010-2019.*

Exposure Setting	**n**	**%**	**n HUS**	**% HUS**
No Ruminant Contact or Exposure	1350	81.3	67	5.0
Live or Work on a Farm with Ruminants Only	88	5.3	6	6.8
Visit a Farm or Other Animal Venue with Ruminants Only	194	11.7	24	12.4
Both Live or Work on a Farm with Ruminants AND Visit a Farm or Other Animal Venue with Ruminants	28	1.7	6	21.4
Total				

Table 3-3. *Association between Exposure Setting and Hemolytic Uremic Syndrome (HUS) Adjusted for Age, Shiga Toxin Gene (stx) Profile, and County Ruminant per Capita— Minnesota, 2010–2019.*

	OR	**95% CI**	
HUS		LCI	UCI
Exposure Setting			
(Reference: No Ruminant Contact or Exposure)			
Live or Work on a Farm with Ruminants Only	1.29	0.53	3.12
Both Live or Work on a Farm with Ruminants AND Visit a Farm or Other Animal Venue with Ruminants	4.03	1.52	10.64
Visit a Farm or Other Animal Venue with Ruminants Only	2.53	1.51	4.25
***stx* Profile**			
(Reference: stx1 & stx2)			
stx2	2.93	1.85	4.66
Age	0.97	0.96	0.98
County Ruminant per capita	0.96	0.83	1.11

Supplementary Material

We imputed 40 datasets, 5 iterations each, to reduce the loss of efficiency to <1%, having confirmed that ≤40% of data was missing for any given variable [33]. The success of the imputation was determined by assessing diagnostic boxplots (continuous variables) and density plots (categorical variables).

Chapter 4 Social Influence Impacts Handwashing Behavior in Adults at Public Animal Contact Venues: A Discrete Choice Experiment

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Introduction

Outbreaks and sporadic cases of zoonotic disease from public animal contact venues, such as fairs, petting zoos, and agritourism operations, have been repeatedly documented in the United States. During 2009-2018, there were 512 reported outbreaks of intestinal disease associated with live animal contact in the United States, resulting in 13,320 illnesses, 2,458 hospitalizations, and 21 deaths [26]. Diseases that can be transmitted at these events include fecal-oral transmitted diseases (e.g., salmonellosis, listeriosis, cryptosporidiosis, and Shiga toxin-producing *E. coli* infection) and diseases transmitted through respiratory droplets or aerosols (e.g., such as influenza, tuberculosis, and Q fever) [33]. People commonly exhibit high risk behaviors at public animal contact events, including touching their faces, making face-to-face contact with animals, eating and drinking in animal areas, contacting animal feces, and having contact with inanimate objects that can be contaminated with animal feces (e.g., strollers, fence rails) [33, 154-155].

It has been well established that handwashing reduces infection risk [37-40, 156-158]. However, observational studies of handwashing at U.S. fairs have demonstrated low handwashing prevalence among fairgoers [154, 159]. Although public health practitioners have made public health guidance readily available for animal event planners and organizers who wish to make changes to facility design at their events, few studies have determined which interventions are most effective at optimizing handwashing behaviors [33].

In this study, we used a discrete choice experiment (DCE) to determine the most effective strategies for increasing handwashing among fairgoers at public animal contact venues. We assessed fairgoers' willingness to practice appropriate handwashing given varying levels of resource accessibility, time availability, consequences, and social influence. We found that social influence and handwashing station availability and accessibility have a significant impact on handwashing behavior.

Materials & Methods

Attribute Selection and Survey Design

This was a cross-sectional study using a convenience sample of fairgoers at the Minnesota State Fair, which took place from August 26 to September 6, 2021. We used a DCE which provided an advantage by allowing the isolation of marginal effects of various factors [160]. The discrete choice questions were designed to examine four attributes determined through a literature review that could impact handwashing in agricultural fairs and other animal contact events. These attributes were: resource accessibility, time availability, consequences, and social influence (**Table 4-1**).

Resource accessibility refers to the accessibility of a handwashing station at the exit of an animal barn. Time availability applies to the amount of time taken to wash hands. Consequences are negative repercussions a person may face from not washing hands. Social influence concerns the social pressures one might or might not encounter in an animal barn. Resource accessibility was measured by varying the distance between the participant and the nearest handwashing station across three levels, and accordingly, the time it took to walk there [161-165]. Time availability was measured by varying the number of people in line in front of the participant for the nearest handwashing station across four levels, and accordingly, the wait time [166]. Consequences were measured by four situations with varying degrees of negative impacts a person would receive from not complying with public health recommendations as indicated by signage [166-168]. Social Influence was measured by four situations that varied in the type of person or group who was relaying a message and whether handwashing was encouraged or discouraged (**Table 4-1**) [161,169-175].

From 192 possible combinations of attribute-specific parameters possible for each question (**Table 4-1**), a fractional factorial design was used to generate 48 scenarios which were paired into 24 choice tasks. These choice tasks were then block randomized into four randomly assigned blocks of six questions each. Twenty-four combinations resulted in a well-balanced fractional factorial with 100% efficiency [176]. Given that the number of choice questions per respondent (NREP) was 6, the number of alternatives per choice set was 2, and the largest number of levels in any attribute was 4, the ideal sample size per block was 167 people, implying a total of 668 people needed to have a well-powered study design [177].

The survey included four demographic questions documenting the respondent's age, gender, race, and ethnicity. Six discrete choice questions followed. Each study participant was presented with the same overall situation; they were asked to imagine that they were at a fair inside an animal barn (Supp.). Within each question, the participant was given two side-by-side scenarios (image-based) and asked to choose in which scenario they would be more likely to wash their hands in a multiple-choice format: Scenario A, Scenario B, or

Neither (**Figure 4-1**). The choice questions were followed by seven questions regarding the respondent's existing practices pertaining to public animal contact venues. Participants were asked if they visited an animal barn that day or in the past and, if so, who they brought with them into the animal barn (age groups), what personal items they brought with them into the animal barn, and whether they washed their hands after leaving.

Surveys were administered over six shifts (30 hours) at the 2021 Minnesota State Fair in the Driven to Discover research facility [178]. Fairgoers were eligible for this study if they were aged 18 years or older and able to read and speak English fluently. Participants signed an electronic consent form, confirmed eligibility prior to participation, and were offered a drawstring backpack as an incentive to participate. Participants completed the electronic survey using iPads. Ethical approval for this study was obtained through the University of Minnesota Institutional Review Board (IRB ID: STUDY00009805).

Statistical Analysis

Random utility theory (RUT) provides the underlying foundation for DCE and operates under the assumptions that individuals make choices that maximize their utility, people have consistent preferences, and that preferences can be transformed into utility structures [179-180]. The generalized utility function (Equation 1) describes the utility that an individual, n, gets from an alternative, j, in choice task t as a function of a deterministic component comprising the attributes' levels and coefficients ($\beta_n \chi_{njt}$) and a random component that the researcher cannot observe (ε_{njt}) [179]. The deterministic component is a function of two vectors: β, which is dependent on factors specific to the individual and χ_{njt}, which is representative of characteristics concerning the specific choice decision presented. The logit model can be used by assuming that each error (ε_{njt}) is an independently, identically distributed (iid) extreme value. This is also referred to as the Gumbel distribution [181].

$$U_{njt} = \beta_n \chi_{njt} + \varepsilon_{njt} \qquad \text{(Equation 1)}$$

The probability that an individual makes a particular decision based on the combined presentation of parameters is calculated by first determining the conditional probability of a person making the entire sequence of choices (Equation 2) and second estimating the unconditional probability by taking a weighted average of logit choice probabilities (weighted by the density of βs) (Equation 3). The resulting unconditional probability can be used to simulate maximum likelihood estimates using mixed logit models [180-181].

$$L_{ni}(\beta) = \prod_{t=1}^{T} [\frac{e^{\beta_n' x_{nii_t t}}}{\sum_j e^{\beta_n' x_{njt}}}] \qquad \text{(Equation 2)}$$

$$P_{ni} = \int L_{ni}(\beta) f(\beta) d\beta \qquad \text{(Equation 3)}$$

A person's willingness to pay (wtp) is defined as the "price" denominated in time at which the respondent is indifferent between washing their hands and not washing their hands. When considering handwashing at public animal contact venues, we included time as a price proxy to estimate the time a person was willing to spend to wash their hands. Both the resource accessibility and time availability attributes had a time component associated with them in the questionnaire (**Table 4-1**). Marginal wtp was calculated individually for each (resource accessibility and time availability) by taking the ratio of the marginal utilities of the attribute and cost (Equation 4). This yields the change in utility per 1 minute change in time spent [182].

$$mWTP_k = -\frac{MU_k}{MU_C} \qquad \text{(Equation 4)}$$

Descriptive statistics were generated to describe the demographic characteristics of the study sample by calculating the frequency and proportion of study participants by age, gender, race, ethnicity, and existing fair practices.

In our primary analysis, we compared the difference in overall willingness to practice handwashing given varying levels of resource accessibility, time availability, consequences, and social influence. Resource accessibility and time availability were fixed in the model while consequences and social influence were included as random coefficients. This was done under the assumption that all members of the public would consistently want to minimize time spent on handwashing (i.e., resource accessibility, time availability) but the effects of consequences and social influence may vary more person to person.

The primary outcome of interest was the respondent's choice of handwashing scenario (Scenario A, Scenario B, or Neither). These data were analyzed using a mixed logit model with the main effects as independent categorical variables using a hierarchical model for individual level heterogeneity. Resource accessibility and time availability were recoded as continuous variables. The standard deviations reported in the model are reflective of the amount of unanimity in the way people feel about a specific attribute. Finally, we calculated marginal willingness to pay for each attribute by cost (resource accessibility and time availability). All data were analyzed using STATA®/IC 16.1 software.

Results

Descriptive Summary of Participants

A total of 913 people participated, with 232 (25.4%) in block 1 (where each block was made up of 6 DCE questions), 222 (24.3%) in block 2, 230 (25.2%) in block 3, and 229 (25.1%) in block 4. With 913 respondents each providing responses to 6 completed choices, 6,434 observations were recorded (913 individuals x 6 choices x 3 options for each choice [Scenario A, Scenario B, and Neither]).

The average respondent age was 42 years, ranging from 18 to 83 years. The majority of study participants were white (80.7%), female (61.6%), and not Hispanic or Latino (86.2%). Of survey respondents, 836 (91.6%) either visited an animal barn the day of survey completion or had visited an animal barn in the past. Of those who reported ever visiting an animal barn, 629 (75.2%) stated that they washed their hands after leaving.

Cell phones (84.1%) and purses or wallets (74.2%) were the topmost indicated items brought into the animal areas. Strollers (5.3%), toys (1.3%), and pacifiers (1.2%) were indicated the least. Of all respondents, 30.4% reported drinking in animal areas (**Figure 4-2**). Most study participants were accompanied by an adult (43.4%) while 17.4% were accompanied by a teenager (13-17 years), 11.0% were accompanied by a child (5-12 years) and 8.3% were accompanied by an infant or toddler (0-3 years) (**Figure 4-3**).

Resource Accessibility and Time Availability

The odds of handwashing were 23% lower for every 1 unit increase in distance to the handwashing station (OR = 0.77; 95% CI: 0.74, 0.80). Handwashing probability was 13% lower for every additional person waiting in line (OR = 0.87; 95% CI: 0.85, 0.89) (**Table 4-2**).

Social Influence

Having a fair staff member direct the fairgoer to the nearest handwashing station significantly increased handwashing probability among survey respondents compared to having nobody around (OR = 1.49; 95% CI: 1.30, 1.72). Marginal willingness to pay estimates indicated that a fair staff member directing the respondent to the handwashing station was the most effective method of increasing the length of time spent on handwashing. Respondents were willing to spend an additional 2.92 minutes waiting in line and an additional 1.51 minutes walking to the nearest handwashing station if a staff member directed them to the nearest handwashing station compared to when nobody was around (**Table 4-3**).

The social influence of other fairgoers social distancing, wearing masks, and washing their hands also increased handwashing probability compared to having nobody around (OR = 1.40; 95% CI: 1.23, 1.60) (**Table 4-2**). Study participants were willing to spend an additional 2.46 minutes of time waiting in line for a handwashing station if everyone around them was masked, socially distant, and washing their hands compared to when nobody was around. They were willing to spend an additional 1.27 minutes walking to the nearest handwashing station under the same conditions (**Table 4-3**).

Social pressure also influenced the choice *not* to wash hands. Social pressure from friends to not wash hands significantly reduced handwashing probability compared to having nobody around (OR = 0.81; 95% CI: 0.70, 0.93) (**Table 4-2**). Study participants preferred to save 1.54 minutes of wait time and 0.79 minutes of walking time if their friends were leaving without washing hands to get food compared to when there was nobody around (**Table 4-3**).

CONSEQUENCES

In our sample, suggesting that failing to wash hands could result in severe illness and even death increased handwashing probability when compared to not having any written consequences on signage (OR = 1.21; 95% CI: 1.06, 1.39). Conversely, indicating that washing hands would prevent the spread of diseases like COVID-19 (OR = 1.01; 95% CI: 0.88, 1.15) and warning that fairgoers may be asked to leave the fair if they did not wash their hands (OR = 0.91; 95% CI: 0.81, 1.04) did not influence handwashing behavior.

Discussion

Handwashing probability was significantly influenced by positive and negative social pressures. We found that having a fair staff member direct study participants to a handwashing station maximized the time spent on handwashing. This was closely followed by the policy of everyone washing their hands, wearing masks, and standing 6 ft. apart. Conversely, social pressure from friends to leave animal areas without washing hands significantly reduced handwashing probability in our sample. We also showed that handwashing probability increases with reductions in walk time and wait time for handwashing facilities. Signage emphasizing the potential for severe clinical illness from improper handwashing was effective at increasing handwashing probability. In this study, survey respondents self-reported exhibiting high risk behaviors in animal areas. Almost a third of respondents reported bringing drinks into the animal barns. It was also common for participants to bring small inanimate objects into animal areas, including cell phones, wallets, and car keys. Objects such as these have the potential for contamination if accessed while interacting with farm animals.

This study provides evidence of the impact that positive and negative social pressures can have on handwashing behavior. The relationship between staff involvement and handwashing has been demonstrated in previous studies. A 2011 observational study demonstrated that personnel involvement (i.e., physically offering hand sanitizer to visitors, verbal reminders to visitors to wash hands) improved handwashing practices [183]. This observation was corroborated in a 2012 study of Kansas and Missouri petting zoos [154]. Our findings are novel in that we found that staff engagement was the strongest predictor of increased handwashing probability compared to other interventions.

We also showed that the negative social pressure of friends encouraging a fairgoer to leave without washing hands reduced handwashing probability in our study participants. Although fewer studies have examined social pressures in the context of handwashing at public animal contact venues, peer pressure has been shown to influence handwashing in various populations globally [184-186].

Finally, we demonstrated that the social influence of other fairgoers wearing masks, socially distancing, and standing 6 ft apart significantly increased handwashing probability. Given the relative novelty of these public health interventions, the influence of others adopting these practices on individual behavior has not been thoroughly studied. However, study recruitment took place in August 2021 and thus selectively included study participants whose risk perception of COVID-19 was low enough that they attended an event averaging approximately 140,000 people per day [187]. It is notable that the social pressure of a masking and social distancing policy was effective at increasing handwashing probability in a sample who was not influenced by the warning that "Washing hands will prevent the spread of diseases like COVID-19" on signage. This implies that social pressures can largely influence the adaptation of public health practices even in a population where risk perception is relatively low.

Our findings also demonstrated that increasing the availability and accessibility of handwashing stations significantly improved handwashing. This aligns with existing evidence in the literature. Petting zoo observation in Ontario, Canada documented that handwashing compliance increased with handwashing station placement along exit routes, increased signage, and running water availability [155]. An observational study of Kansas and Missouri petting zoos noted that handwashing signs and hand hygiene stations were placed upon leaving 77% of animal areas and 62% of events [154]. Although 75.2% of participants in our study self-reported that they washed hands after leaving animal areas, this proportion was much higher than estimates from observational studies at other U.S.

events [154].The importance of handwashing station visibility in exit transition areas and staff involvement at events are highlighted along with other recommendations in the National Association of Public Health Veterinarians Compendium of Measures to Prevent Disease Associated With Animals in Public Settings [33].

Our findings have implications for facility design at public animal contact venues. Wait times, and especially walk times, need to be reduced at events to compensate for the social pressures of others not washing hands and/or encouraging others not to spend time washing hands. Facilities should consider staff engagement or recruiting volunteers to remind event guests to wash hands. Displayed signage should also emphasize the severity of clinical illness that can ensue if a fairgoer does not wash their hands. Managing the consumption of beverages in animal areas may be an area of focus for prevention efforts. The successful enforcement of public health policies could be effective at influencing handwashing among fairgoers.

Our sampling plan may have impacted the generalizability of our results. Completing the survey may have been a time inconvenience to fairgoers with young children. Our sample selectively included participants who attended the Minnesota State Fair despite a high risk of COVID-19 transmission. These same participants were also willing to wear a mask during survey completion, which was a requirement in the building where surveys were disseminated. These biases may have influenced our model estimates; particularly, the estimate describing the association between signage warning that "washing hands will prevent the spread of diseases like COVID-19" and handwashing probability may have been overly conservative.

These findings support ongoing efforts to protect the public at animal exhibiting and animal contact events. Because of the increased risks to children and the elderly, additional measures are needed to reduce these risks. Understanding social behaviors are key to accomplishing hazard reduction. We recognize that specific interventions that maximize handwashing are beneficial for venues. Often these venues are under-resourced or have limited public health interventions. These findings in addition to current guidance documents can help venue operators recognize and reduce potential zoonotic risks to the public.

Acknowledgments. The authors would like to acknowledge Anne Marie Hoptop and the Driven to Discover Research Facility at the Minnesota State Fair for allowing us to disseminate surveys and conduct research at their facility. We would especially like to acknowledge the volunteers who worked to recruit and enroll study participants: Brenna Horn, Hanna Root, Jennifer Koper, Kianna Dufault, Maryam Zahedi, Navid Emamdoost, Nicholas Vachon, Teddy Nam, and Thuy Kim.

Tables

Table 4-1. *Discrete Choice Experiment Attributes and Levels*

Resource Accessibility	**Distance:** Handwashing is 3 ft away (< 1 min. walk) **Distance:** Handwashing is 500 ft away (2 min. walk) **Distance:** Handwashing is 1000 ft away (4 min. walk)
Time Availability	**Time:** There is 1 person in front of you (<1 min. wait) **Time:** There are 4 people in front of you (2 min. wait) **Time:** There are 6 people in front of you (3 min. wait) **Time:** There are 12 people in front of you (6 min. wait)
Consequences	**Consequences:** "Please wash your hands after leaving the animal barns." **Consequences:** "Please wash your hands after leaving the animal barn. Washing hands will prevent the spread of diseases like COVID-19." **Consequences:** "Please wash your hands after leaving the animal barns. Failing to wash your hands can result in severe illness and even death." **Consequences:** "Please wash your hands after leaving the animal barns. If you do not wash your hands, you may be asked to leave the fair."
Social Influence	**Social Influence:** There is nobody around you who you know **Social Influence:** A fair staff member directs you to the handwashing station as you're leaving the animal barn. **Social Influence:** Everyone around you is washing their hands, wearing masks, and standing 6 ft apart. **Social Influence:** Your friends leave without washing their hands. They say, "Come on, let's get food!"

Table 4-2. *Estimates of attribute-specific parameters associated with willingness to wash hands*

Attributes	**OR**	**95% CI**	
Resource Accessibility	0.766	0.737	0.797
Time Availability	0.872	0.850	0.894
Consequences *(Reference: No Consequence)*			
Washing hands will prevent the spread of diseases like COVID-19	1.006	0.879	1.151
Failing to wash your hands can result in severe illness and even death.	1.214	1.062	1.388
If you do not wash your hands, you may be asked to leave the fair.	0.914	0.806	1.036
Social Influence (Reference: There is nobody around you who you know)		1.299	1.719
A fair staff member directs you to the handwashing station as you're leaving the animal barn.	1.494		
Your friends leave without washing their hands. They say, "Come on, let's get food!"	0.809	0.704	0.931
Everyone around you is washing their hands, wearing masks, and standing 6 ft apart. /Normal	1.402	1.231	1.597
sd(Washing hands will prevent the spread of diseases like COVID-19)	0.648	0.440	0.954
sd(Failing to wash your hands can result in severe illness and even death.)	0.905	0.704	1.163
sd(If you do not wash your hands, you may be asked to leave the fair.)	0.640	0.444	0.922
sd(A fair staff member directs you to the handwashing station as you're leaving the animal barn.)	0.972	0.766	1.234
sd(Your friends leave without washing their hands. They say, "Come on, let's get food!")	0.945	0.755	1.182
sd(Everyone around you is washing their hands, wearing masks, and standing 6 ft apart.)	0.823	0.627	1.080

Table 4-3. *Marginal Willingness to Pay for Each Attribute Relative to Resource Accessibility (i.e., time spent walking to handwashing station) and Time Availability (i.e., time spent waiting in line for a handwashing station)*

Resource Accessibility	
Attributes	**mWTP**
Consequences *(Reference: No Consequence)*	
Washing hands will prevent the spread of diseases like COVID-19	0.02
Failing to wash your hands can result in severe illness and even death.	0.73
If you do not wash your hands, you may be asked to leave the fair.	-0.34
Social Influence (Reference: There is nobody around you who you know)	
A fair staff member directs you to the handwashing station as you're leaving the animal barn.	1.51
Your friends leave without washing their hands. They say, "Come on, let's get food!"	-0.79
Everyone around you is washing their hands, wearing masks, and standing 6 ft apart.	1.27
Time Availability	
Consequences *(Reference: No Consequence)*	
Washing hands will prevent the spread of diseases like COVID-19	0.04
Failing to wash your hands can result in severe illness and even death.	1.41
If you do not wash your hands, you may be asked to leave the fair.	-0.66
Social Influence (Reference: There is nobody around you who you know)	
A fair staff member directs you to the handwashing station as you're leaving the animal barn.	2.92
Your friends leave without washing their hands. They say, "Come on, let's get food!"	-1.54
Everyone around you is washing their hands, wearing masks, and standing 6 ft apart.	2.46

Figures

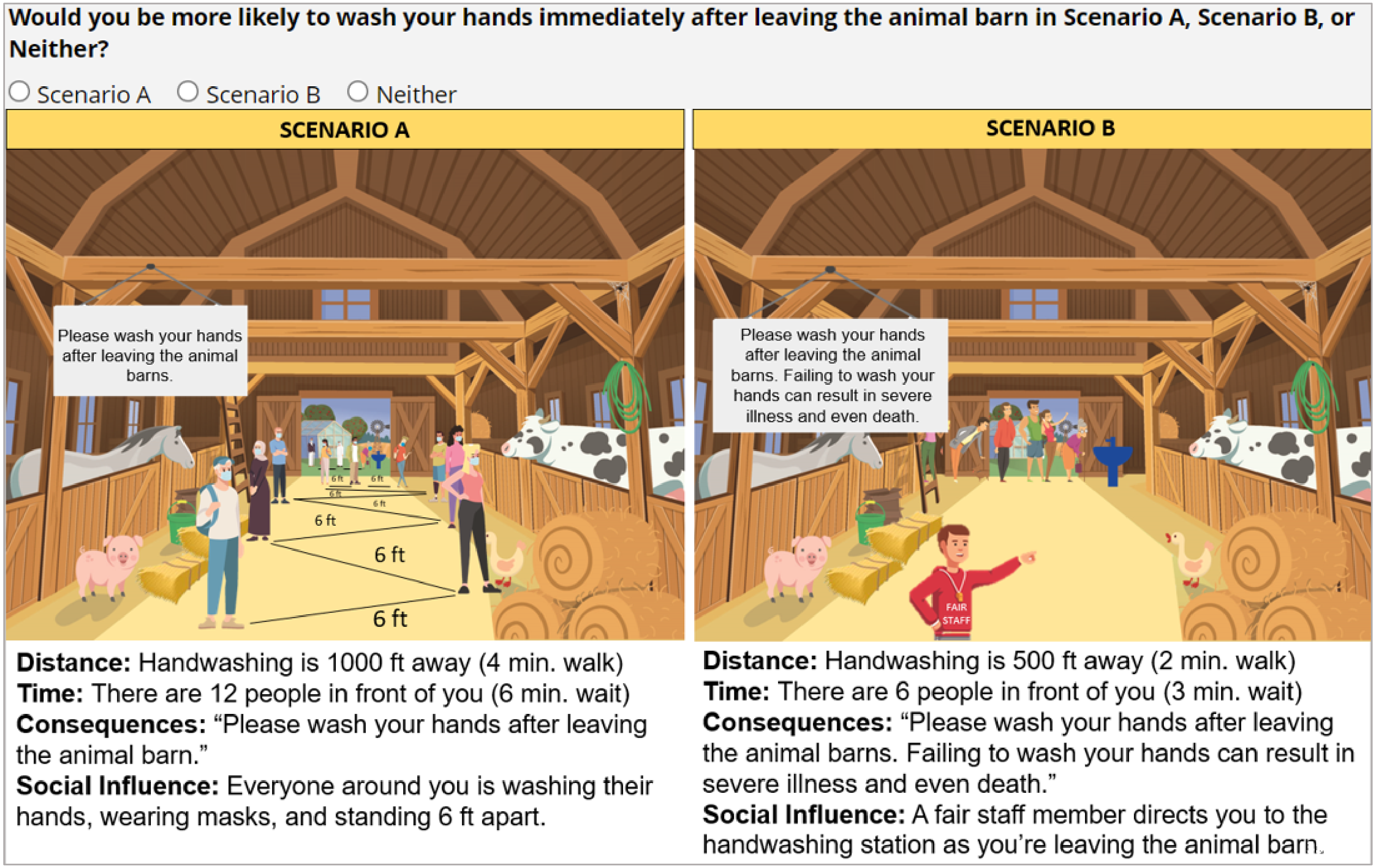

Figure 4-1. *Example Discrete Choice Question*

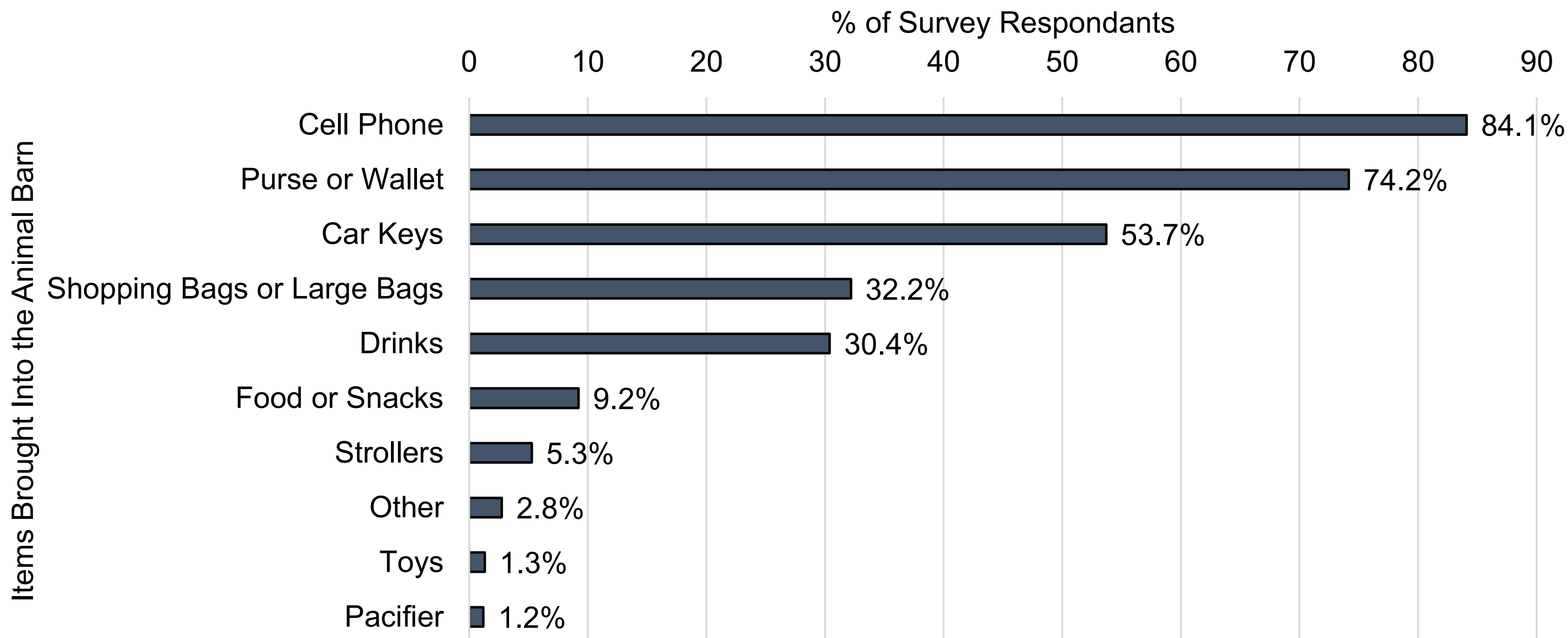

Figure 4-2. *Bar chart of personal items brought into the animal barn among survey respondents who visited an animal barn on the day of survey completion or who reported having visited an animal barn in the past*

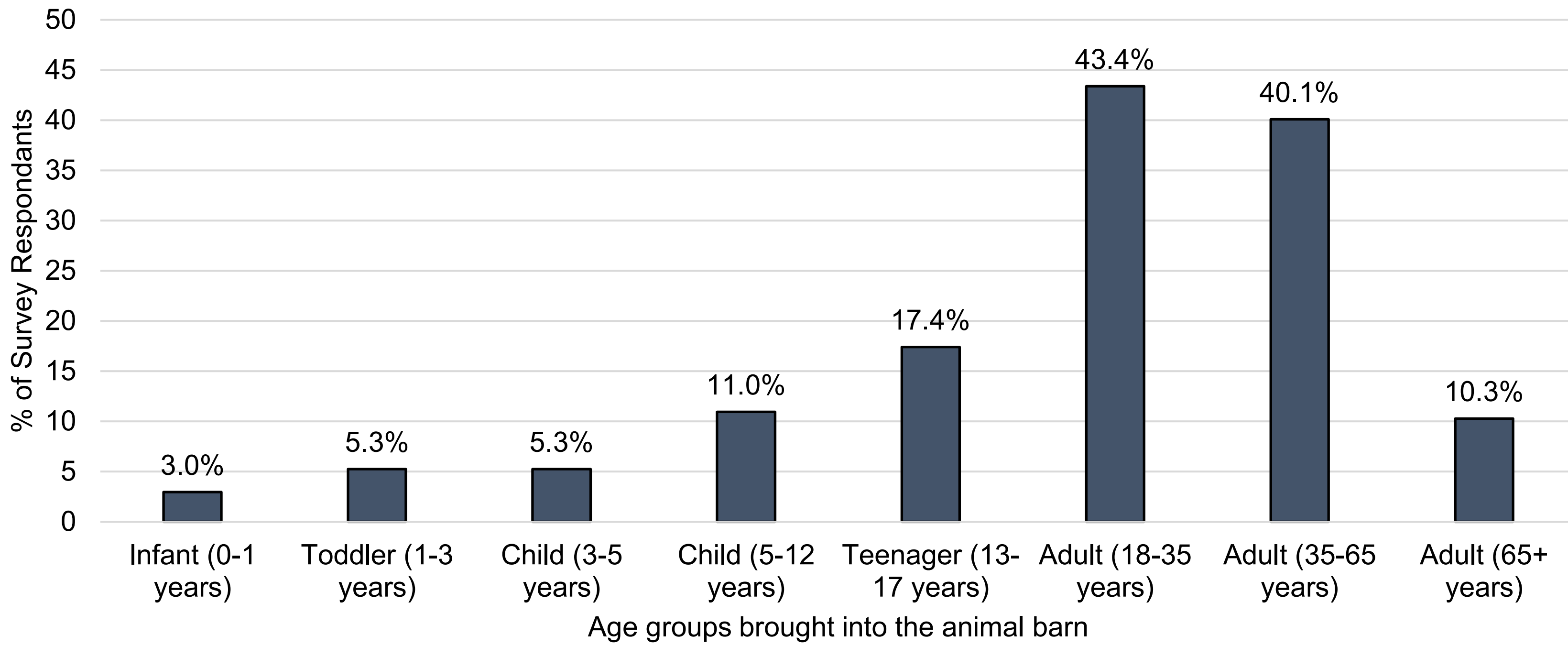

Figure 4-3. *Bar chart of age groups brought into the animal barn among survey respondents who visited an animal barn the day of survey completion or who reported having visited an animal barn in the past.*

__Supplementary Material__

Overall Situation

INSTRUCTIONS:

For the next 6 questions, imagine you are at a fair inside an animal barn (as shown by the images that are meant to be from your perspective).

Two options will be given to you. The following things will change slightly each time:

Resources: How far away are you from the nearest handwashing station (any sink with soap and paper towels)?
Time: How many people are in line in front of you for the nearest handwashing station?
Consequences: What warnings are on the sign in the animal barn?
Social Influence: What are the social influences around you?

Please decide __**in which option would you more likely wash your hands after leaving the animal barn.**__

The questions change slightly each time, so please ***read everything carefully*** *and* ***__answer honestly__***!

__**This is a questionnaire is about your opinions; there is no right or wrong answer**__

Figure S-4-4. *This is the overall situation that was presented to all survey respondents prior to answering 6 discrete choice questions.*

Chapter 5 Stress levels in Exhibition Cattle: a pilot study —Minnesota, 2022

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Introduction

Acute stress in cattle can negatively impact animal health, human health, and both the quantity and quality of agricultural products. Studies have shown that stress increases the risk of infection, weakens immune response, and contributes to weight loss in cattle [188-191]. This includes reduced feed intake, low energy, conception rates, milk production, and increased premature births [192-195]. The quality of beef and dairy products can be negatively impacted by various stressors [65, 196-197]. Additionally, stress in cattle increases the risk of bacterial shedding into the environment, which poses a human health risk [55, 60-63, 66, 73, 152, 198].

Cattle experience psychologic stress from handling, transportation, commingling or social mixing, exposure to new and unfamiliar environments, exposure to loud or unfamiliar noises, and physical restraint or stimulus [67, 199-200]. All of these are common at agricultural fairs and other cattle shows. However, existing literature evaluating cattle stress has focused on transportation and on slaughter-specific stressors. Stress studies have not quantified cortisol levels at agricultural fairs where environmental stimuli are different and the contact rate between farm animals and members of the public is high. Also, there may be mitigating factors with handling and training of exhibition animals.

To address this knowledge gap. we carried out a prospective cohort study in Minnesota cattle to compare stress, as measured by serum cortisol levels (ng/mL), in show cattle with matched cattle at the same farm location that were not being shown.

Materials and Methods

Participant Recruitment and Enrollment

Cattle owners were recruited to participate in this study via a recruitment flyer and corresponding letter (Supp.). Both materials were disseminated to Minnesota Future Farmers of America Association (MN FFA) teachers in their monthly newsletter [201] and via email. The Minnesota Dairy Initiative also disseminated the recruitment materials to farmers on their listserv [202].

Cattle owners were eligible to enroll their cattle in the study if they were showing cattle at a county or state fair in Minnesota in the summer of 2022. Study participants completed a 38-question survey that included questions about basic farm-specific information, perceived stress in their cattle, history of showing animals, show preparation activities, and cattle transportation, feed, and lodging. The survey portion of this study was approved by the University of Minnesota Institutional Review Board (IRB ID: STUDY00015655). The cattle testing portion of this study was approved by the Institutional Animal Care and Use Committee (Protocol number: 2201-39750A).

Study Design

Blood samples were collected first from both index (show cattle) and reference (farm cattle) at farm locations prior to transportation to the show (T1). Samples were collected again at a second time point, from both index and reference cattle, after arrival of index cattle to the fair (T2). For two pairs of beef cattle, samples were collected the day after arrival to the fair due to a scheduling conflict. Samples were collected at a third time point, from both index and reference cattle, on the day of departure of index cattle back to the farm. This last sample was taken after cattle shows were completed and prior to departure from the fair (T3) (**Figure 5-1**). All samples from reference cattle were taken at the home farm location on the same day and within the same time frame that index cattle were sampled. The average time between index and reference cattle sampling was 50 minutes. Two time points were used at the fair to distinguish between the stress associated with transportation (T2) and the stress associated with fair activities (T3). There was an average of 8.25 days between T1 and T2 and an average of 3.75 days between T2 and T3.

SAMPLE COLLECTION

Blood samples from eight pairs of cattle were collected for this study. Serum was used for measuring cortisol levels (as opposed to feces or hair) because it was necessary to determine the cross-sectional measure of cortisol specifically at the time point of collection. Serum collection was also more convenient than saliva collection. Blood was collected into red top tubes by coccygeal venipuncture to avoid the need for head restraint [203-205]. The blood was spun in a variable speed centrifuge at 2,000xg (Medicus Health Item Number: 5261M1) on site within one hour of collection to separate the serum from the red blood cells (**Figure 5-2**) [203]. All samples were collected between 15:00 and 20:00 because the normal diurnal cycle of cortisol shows that cortisol levels spike during the awakening period and decline throughout the day. Therefore, the afternoon sampling time frame would have less of the natural variation in cortisol levels [206]. However, the diurnal cycling of cortisol may not be as pronounced in ruminants, which allowed us more flexibility in our sampling window [207-208]. Tubes were labeled with the cow or calf's identifying information [Cattle ID, Date/Time of collection, Timepoint (T1, T2, or T3)] and transported on ice to the Stepanov lab at the Cardiovascular and Cancer Research Building (CCRB) where the cortisol biomarker was analyzed.

Cortisol Analysis

The frozen serum and blood samples were separated and aliquoted from the red blood cells and clot before storing in a -20^{o}C freezer. The cortisol extraction utilized for this study has been described in detail by Domenech-Coca, et al. in 2019 [209]. In brief, the thawed serum samples were mixed with a known concentration of isotope-labeled internal standard, $^{13}C_3$-cortisol, (Millipore-Sigma: C-216) for quantification with a standard curve. Cortisol was extracted using three rounds of ethyl-acetate liquid-liquid extraction. The supernatants were pooled together, evaporated to dryness, and reconstituted in 40% methanol. The extract was analyzed by HPLC-MS/MS using a method optimized by the Stepanov lab to quantify the mass-to-charge ratio (m/z) transitions for cortisol (363.26 → 121.10) and $^{13}C_3$-cortisol (366.26 → 124.10). The serum cortisol levels (ng/mL) were calculated using a cortisol standard curve that was run concurrently with the cattle samples.

Statistical Analysis

A descriptive analysis of the survey results was performed. Descriptive statistics included frequency analysis (percentages) for categorical variables and mean and standard deviation or median and interquartile range for continuous variables. Our primary outcome of interest in this study was serum cortisol level (ng/mL), a continuous outcome. Bivariate comparisons were made between predictors of interest (timepoint of collection, age, distance traveled to the fair, cattle type, and farm number) and serum cortisol levels. We generated a correlation coefficient for continuous predictors of interest (age, distance traveled). A two-sample t-test was used for binary categorical predictors (cattle type). A one-way analysis of variance (ANOVA) was used for categorical predictors with three or more levels (timepoint of collection, farm number). We compared serum cortisol levels of show cattle with matched cattle from the same farm location by performing a paired t-test at each time point. All data were analyzed using R (version 4.0.3, R Core Team, 2020).

Results

Survey Results

Four youth cattle exhibitors who owned the tested cattle completed surveys for this study (**Table 5-1**). All survey respondents perceived stress in their cattle in some way. The most commonly noted stress behaviors included vocalizing, open mouth breathing, restlessness or agitation, and reduced feed intake. All respondents observed stress in their animals when surroundings were chaotic (**Table 5-2**). Respondents noted that providing cattle with an electrolyte pack and applying topical pheromone, petting/scratching the neck/tail region, and speaking to cattle in a calming voice were all successful strategies to lower cattle stress.

The most common show preparation activities were grooming/brushing/combing cattle, washing cattle with soap or conditioner, putting a halter on cattle, walking cattle, and setting up their stance with hands or feet (**Table 5-2**). Show cattle were given feed on average 1.7 times a day while at the farm and an average of 2.3 times a day while at the show. The majority of cattle were fed hay. Cattle were also fed prepackaged specialized show feeds, vitamins or other supplements, corn, wheat, and total mixed rations (TMR) (**Table 5-2**).

Most respondents reported transporting cattle in a gooseneck stock trailer with no ramp (75%), while 25% reported having a bumper pull stock trailer. The maximum number of cattle transported within the same vehicle was 10, where each animal was allotted 134ft^3. Show cattle spent an average of 70% of time outdoors in the two weeks prior to show.

Cortisol Results

There were 45 observations in the final dataset; samples were missing from three cattle, all of which were at T3. Blood was collected using two tubes for one cattle in this study; the resulting cortisol measurements from each tube were averaged to generate a final result. There were 16 total cattle in this study (8 pairs) from three separate farms (**Table 5-1)**. The majority of cattle were dairy (75%) and the remaining 25% were beef cattle. Median cattle age was 12 months and ages ranged from 3 to 72 months (**Table 5-1**).

There were two dairy farms included in this study, one with approximately 500 cattle and one with approximately 200 cattle. The third farm was smaller and housed 7 beef cattle.

Average serum cortisol level among all cattle was 5.0 ng/mL with a minimum of 0.39 ng/mL and a maximum of 22.17 ng/mL. Average serum cortisol levels were highest at T2 (6.18 ng/mL) (**Figure 5-3**). However, we observed no difference in mean serum cortisol levels over all cattle across all three time points (F=0.81, p=0.5). Serum cortisol levels were consistently higher in farm cattle when compared to show cattle (**Figure 5-4**). Average serum cortisol levels among index (show) cattle at T1 was 3.15 ng/mL (Range: 0.39-10.97 ng/mL) and among reference (farm) cattle at T1 was 4.67 ng/mL (Range: 0.71-9.46 ng/mL). Average serum cortisol levels among index cattle at T2 was 3.70 ng/mL (Range: 0.95-7.54 ng/mL) and among reference cattle at T2 was 8.67 ng/mL (Range: 0.83-22.17 ng/mL). Average serum cortisol levels among index cattle at T3 was 4.09 ng/mL (Range: 0.61-9.25 ng/mL) and among reference cattle at T3 was 7.92 ng/mL (Range: 0.55-17.53 ng/mL). We observed no difference in mean serum cortisol levels between index and reference cattle at any time point (**Table 5-3**). There was a weak negative relationship between cattle age and serum cortisol levels (R=-0.24). Beef cattle had higher average serum cortisol levels (7.3 ng/mL) than dairy cattle (4.3 ng/mL), however, this difference was not significant (t=1.33, p=0.21). We observed no difference in average serum cortisol levels by farm (F=1.55, p=0.22).

Discussion

Mean cortisol levels between show and matched farm cattle were not significantly different at any time point. The three outliers in this study (**Figure 5-3**) all required wrangling prior to testing; the resulting increase in cortisol may have been due to the unfamiliar surroundings and events that went into sample collection. Cattle owners self-reported that show cattle received extra attention. When handlers observed stressful behaviors in their cattle, their handling and reassurance may have mitigated stress which is suggestive of a pathway to improving the safety of animal contact venues.

Mean serum cortisol levels from all cattle in this study (5.0 ng/mL) were lower than established mean basal serum cortisol concentrations in unstressed cattle from prior studies (7.25 ng/mL) [210]. Serum cortisol levels from all cattle in this study were generally lower than levels reported in other stress studies. This may be because cattle are sampled when exposed to harsher environments than typically associated with a cattle show, including longer transportation routes, transportation with many other unfamiliar animals, and slaughter or marketing-related stressors. A 2005 study of 10 cattle in Europe observed that mean serum cortisol levels were 26 ng/mL after being transported 28-40 miles and increased to 35.8 ng/mL after being transported 1367 miles [211]. In our study, the furthest show cattle were transported was 47.2 miles and the highest serum cortisol value among show cattle after transportation was 7.54 ng/mL. Cattle in both studies were given approximately the same amount of space (19 ft^2). It is unclear from the European study whether cattle were familiar with one another prior to transport. Mean plasma cortisol concentrations in a 2006 study of calves aged four months reached 55 ng/mL the day after two-hour transport in a truck [212]. While these calves were taken from different breeding environments and transported together, the three-month old beef calf in our study was transported with its mother as a cow/calf pair with no other animals present. Mean cortisol levels reported from commercial abattoirs reach upwards of 44.6 ng/mL [213-214]. Stress associated with marketing has also been linked to increased bacterial shedding in cattle [152]. However, cattle in slaughter facilities may be subject to electrical prodding, over handling and mishandling, loud and disruptive physical environments, and high killing rates per day. Similarly, stressors encountered during marketing may include feed and water deprivation, castration, or dehorning [61-67, 215]. These risk factors are rarely observed at fairs.

Show cattle in our study had consistently lower serum cortisol levels when compared to matched cattle from the same farm. This may be because show cattle are regularly handled, are used to halters, chutes, grooming and washing activities, walking with other people, and interaction with other people to practice setting up stance and/or profiling. Although stress did increase over time in show cattle, the difference was minimal. From our observations during serum testing, show cattle were easier to maneuver in and out of chutes to complete blood draws for this study while non-show cattle required more wrangling. These observations are consistent with findings that contact with unfamiliar people and chaotic surroundings can lead to acute stress in cattle [65-67]. Calming and soothing activities performed by cattle owners may mitigate acute stress in cattle. The pilot data collected from this study offers potential avenues for future research: a) determining whether the presence or absence of an owner influences serum cortisol levels at animal contact events and b) determining whether cortisol levels fluctuate as new animals are introduced.

Reviews have generalized that stress from transportation may increase bacterial shedding at agricultural fairs [33]. However, we may be overestimating the influence of shedding specifically due to stress at cattle shows given the minimal external stimuli and the large amount of preparatory activity that goes into caring for show animals. Increased disease incidence at animal contact venues may be better explained by the increased bacterial diversity that results from commingling a variety of animals at a single location, human behaviors and implementation of public health preventive measures, or other factors related to exhibition such as different feed sources and changes in eating patterns [33, 153].

Certain logistical factors impacted data quality in this study. Due to the need to work around farm schedules, the four beef cattle in this study were sample the day after transportation to the fairgrounds. This could have impacted serum cortisol levels and is less useful to make direct comparisons. Additionally, some cattle required wrangling in order to collect samples. The stress associated with wrangling procedures may have contributed to fluctuations in serum cortisol. The small sample size in this study limited our ability to apply mixed effects modeling approaches which would have allowed us to adjust for covariates of interest and account for both within-cow and between-farm variability of serum cortisol levels. Using estimates from the literature and assuming a significance level of $\alpha=0.05$, we predetermined that we would have 80% power to detect a change in serum cortisol from 7 to 30 ng/mL if we enrolled 8 pairs of cattle. Given that differences between index and reference cattle in this study were much smaller, we have insufficient power to make meaningful inferences from the data generated. However, overall trends in the data collected have value, particularly the low serum cortisol levels overall, and offer hypotheses for future testing.

<u>Tables</u>

Table 5-1. *Summary of Cattle Pairs Included in Pilot Study by Owner, Age, Farm, and Distance from Farm to Show*

Owner Number	Pair Number	Cattle Type	Age Reference Cattle [FARM] (In Months)	Age Index Cattle [SHOW] (In Months)	Farm Numbe r	Distance from Farm to Show (In Miles)
1	**1**	DAIRY	14	16	1	3.7
1	**2**	DAIRY	6	6	1	3.7
2	**3**	BEEF	48	72	2	40.7
2	**4**	BEEF	3	3	2	40.7
3	**5**	DAIRY	24	24	3	47.2
4	**6**	DAIRY	24	24	3	47.2
4	**7**	DAIRY	10	10	3	47.2
4	**8**	DAIRY	9	9	3	47.2

Table 5-2. *Descriptive Summary of Survey Results*

Stress Perception	
Stress Behavior	**Survey Respondents who Observed this Behavior in their Animals n(%)**
Vocalizing	3 (75)
Breathing with Mouth Open	2 (50)
Restlessness or Agitation	2 (50)
Reduced Feed Intake	2 (50)
Standing while other cattle are lying down	1 (25)
Shaking or trembling	1 (25)
Slobbering	1 (25)
Reduced Milk Production in Cows	1 (25)
Grinding Teeth	1 (25)
Reduced Water Intake	1 (25)
Circumstances During Which Stress Was Observed in Show Cattle	**Survey Respondents who Observed Stress in their Animal During this Circumstance n(%)**
When things around them are chaotic	4 (100)
When they hear loud noises	2 (50)
When they are getting off the trailer after transport to a show	1 (25)
When they are around other people at a fair that are unfamiliar to them	1 (25)
During show activities	1 (25)
When they are separated from non-show cattle in their same herd	1 (25)
During training, setting up stance, profiling	0 (25)
When they are boarding the trailer for transport to a show	0 (--)
When they are around other animals at a fair that are unfamiliar to them	0 (--)
When people try to pet them	0 (--)
Show Preparation	
Show Preparation Activity	**Survey Respondents who Perform This Activity with their Show Cattle**
Groom/Comb/Brush them	4 (100)

Wash them with soap/conditioner	4 (100)
Put a halter on them	4 (100)
Walk them	4 (100)
Set up their stance with your hands or feet	4 (100)
Wash them with water	3 (75)
Change their feed	2 (50)
Blow dry their hide	1 (25)
Use a stick to set up their stance	1 (25)
Profile their legs	1 (25)
Cold boxing	1 (25)
Use electric prods	0 (--)
Feed	**Survey Respondents who Fed this to their**
Hay	4 (100)
Specialized Show Feeds Prepackaged	2 (50)
Vitamins or Other Supplements	2 (50)
Corn	2 (50)
Total Mixed Rations (TMR)	2 (50)
Wheat	1 (25)
Barley	0 (--)
Corn Silage	0 (--)
Haylage	0 (--)
Pasture	0 (--)

Table 5-3. *Paired t-test comparing difference in mean serum cortisol levels between matched show and farm cattle by timepoint*

	Mean of the Differences	**t-value**	**p-value**
T1	-1.15	-0.69	0.51
T2	-5.38	-1.53	0.17
T3	-2.46	-1.03	0.36

Figures

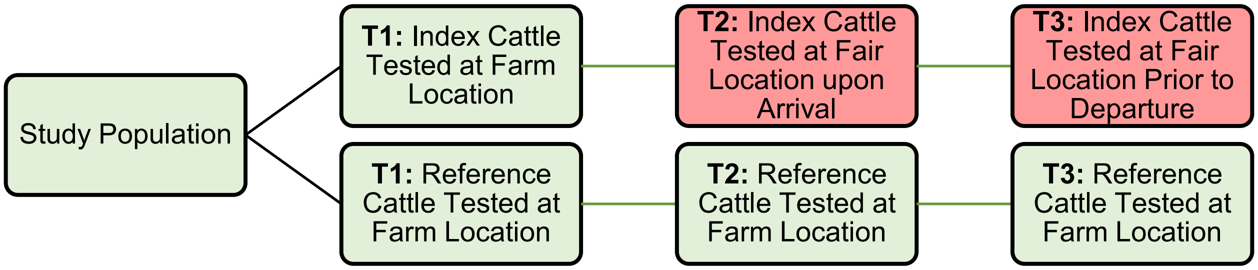

Figure 5-1. *Study Design*

Figure 5-2. *Images of Sample Collection.*

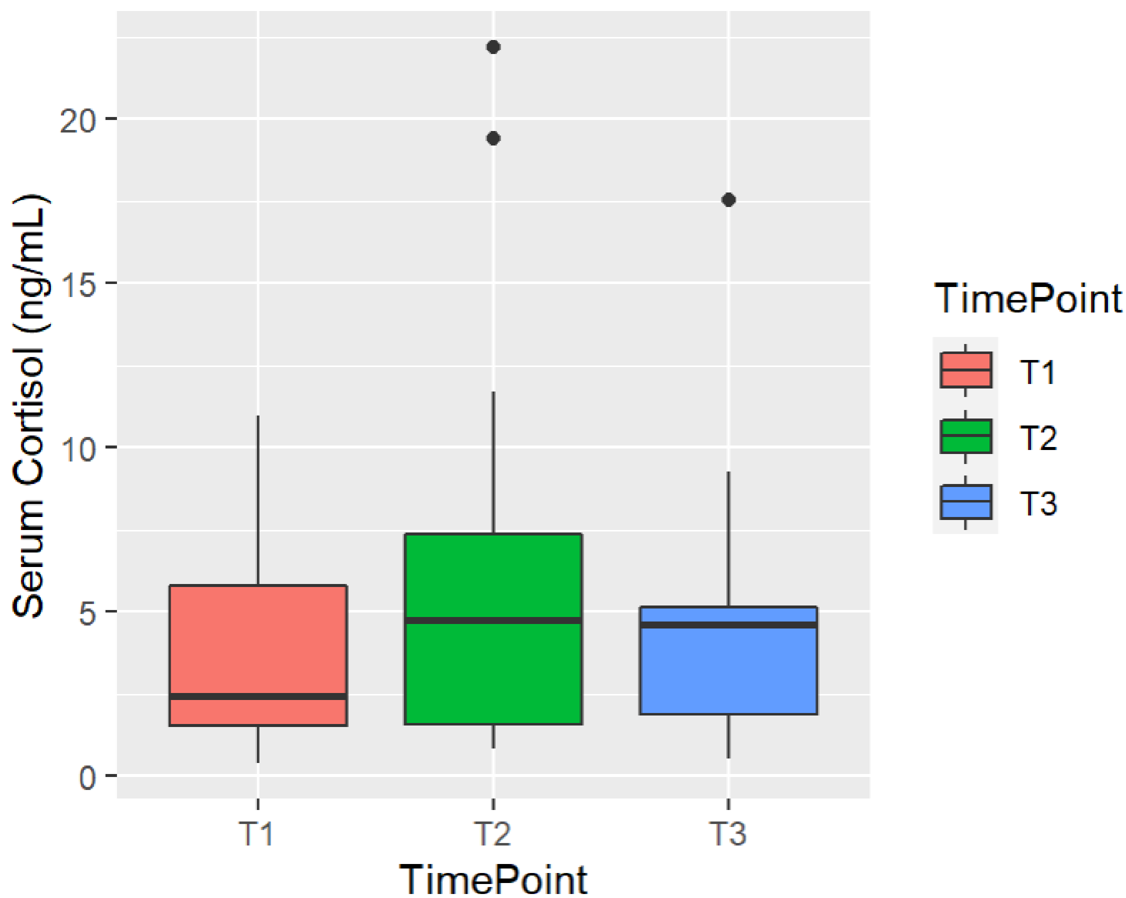

Figure 5-3. *Side-by-side boxplot of serum cortisol levels by time point, All Cattle.*

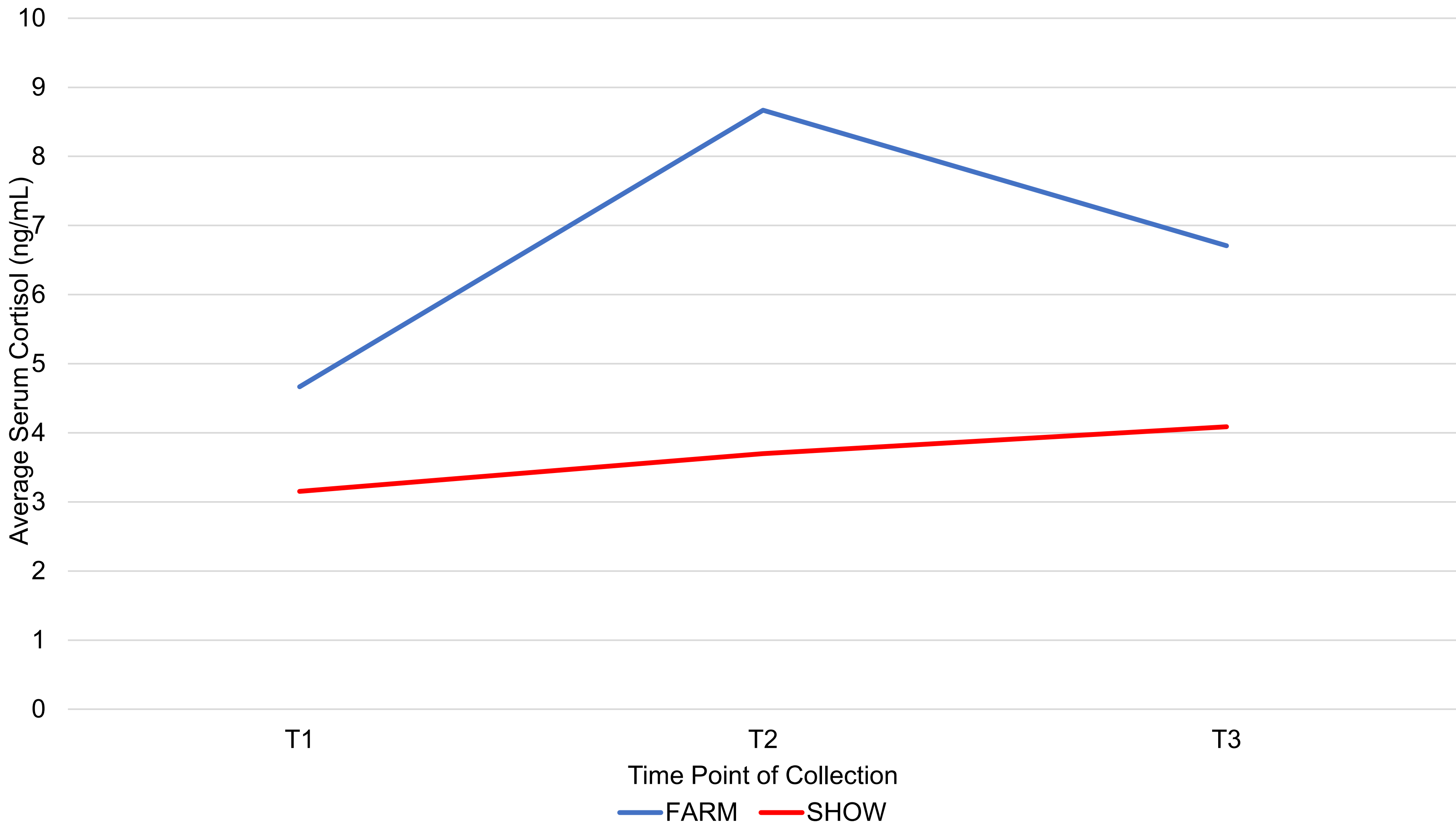

Figure 5-4. *Average Serum Cortisol Level Across Three Time Points by Exposure (Show Cattle/Farm Cattle)*

Chapter 6 Conclusion

Contributions Made From This Work

The aim of this dissertation was to mitigate STEC and HUS risk at animal contact venues simultaneously across the three domains of human health, animal health, and environmental health. The findings within these studies are applicable to clinicians, public health workers, farmers, fair planners and organizers, and members of the public.

Chapter 2, a preface chapter, introduces a novel finding that the source of exposure to STEC can have implications on virulence. Although the relationship between farm animal exposure and STEC incidence has been well defined, this is the first study to propose an independent relationship between farm animal exposure and HUS development. This finding has importance for the clinical management of patients, particularly young children, who present with diarrheal illness and report exposures to farm animals and livestock. This study also emphasizes to clinicians the importance of timely reporting of HUS to facilitate both public health outbreak investigations and the investigation of sporadic cases. The emphasis this study places on the role of the exposure source should compel clinicians to work efficiently with public health colleagues towards rapid source identification. Although guidelines for preventing STEC transmission at animal contact venues have been developed, outbreaks linked to these events continue to occur. The results from this study suggest that we have likely underestimated the risk from these exposures and overestimated the ability of existing guidelines to prevent these exposures.

Chapter 3 further dissects the relationship between the environmental setting where a person was exposed to farm animals and livestock and HUS development. The finding that visiting a farm or other animal venue is associated with HUS development is of particular importance for the farming community. Children living on farms are still at risk of HUS development from visiting other venues and should take care to practice appropriate handwashing and avoid high risk exposures at these events. The age specific patterns observed in this study (increased HUS among young children and older adults) is reflective of the impact that underdeveloped or declining immune mechanisms have on HUS development. Venue operators have a duty to inform the public that human-animal interactions can lead to severe consequences at their events. This study also has use for public health workers. Epidemiologic investigations of common-source outbreaks linked to a single location with suspected animal contact may consider including history of animal exposure on investigation questionnaires. Often, such outbreak investigations do not document individual case histories with respect to animal contact since an epidemiological link between cases has been confirmed. However, detailed exposure histories would expand existing knowledge about hospitalization and HUS development among STEC confirmed cases.

Chapter 4 informs fairs how and where to prioritize interventions. This study sheds light on the power that social influences can have in affecting handwashing probability among fairgoers, particularly in a sub-population where disease risk perception was relatively low. Events with limited public health interventions in place should prioritize involving fair staff in directing fairgoers to handwashing areas. The finding that 30% of fairgoers self-reported consuming beverages in animal areas offers a potential point of intervention at events. This study also highlighted that fairgoers prefer to minimize time spent on handwashing. Therefore, improving the visibility and accessibility of handwashing stations is crucial to promoting handwashing at animal contact venues and compensate for negative social pressures a fairgoer may face.

Chapter 5 findings suggest that results from prior cattle stress studies may not be generalizable to agricultural fair environments given inherent differences in the way cattle are handled and treated for shows compared to other settings like slaughter or marketing. Cortisol levels in tested cattle were generally low, especially compared to other estimates in the literature. The handling, grooming, and preparatory activities that go into getting cattle ready for shows may familiarize them with external stimuli prior to the showing event, thus reducing acute stress in the animal. Although there was limited power in the analysis, data trends and observational elements from this pilot study offer avenues for future research.

Existing Gaps and Avenues for Future Research

Although this work provides evidence of a relationship, the mechanism by which farm animal exposure increases HUS risk is still unknown. Quantification and comparison of exposure dose from ruminant animals, contaminated food, water, and environmental sources is needed to determine how strong of a role dose plays in influencing clinical severity. Analysis of ruminant feces at animal contact venues would also allow researchers to determine pathogen load and obtain a distribution of virulence factors present at these events. Feces analysis prior to the event and following commingling would also shed light on the role that bacterial diversity may play in illness severity. A higher-powered study is needed to concretely determine whether cattle stress from agricultural fairs can be directly compared to stress from other environments where stress studies have been conducted previously. Observations from the cattle study suggested that cattle that did not experience as much handling exhibited more stress from sample collection. Future research should examine the relationship between stress and shedding as new animals are entered into a fair or petting zoo environment to account for stress associated with unfamiliar surroundings and external stimuli. The cattle study also demonstrated that cattle owners sooth and calm cattle that exhibit stress. It would be useful to know whether the presence or absence of an owner adequately influences cattle stress as a potential route of intervention at events like fairs and petting zoos.

Although this work has focused on STEC transmission and HUS development, it can be used as a model for the study of other zoonotic pathogens that circulate at animal contact venues such as salmonellosis, campylobacteriosis, influenza, and tuberculosis. By assessing the burden of illness both at an individual and a systems level, this work provides a comprehensive assessment of STEC transmission at animal contact venues, offers approaches to mitigate risk at these events, and outlines goals for future research to minimize morbidity and mortality in the public.

References Cited

1. Centers for Disease Control and Prevention. (2014). *E. coli (Escherichia coli)* Questions and Answers. Retrieved from: https://www.cdc.gov/ecoli/general/index.html. (accessed 13 November 2019).
2. Konowalchuk, J., Speirs, J.I., Stavric, S. (1977). Vero response to a cytotoxin of Escherichia coli. *Infect Immun*, 18:775–9.
3. Karmali M.A., Steele B.T., Petric M., Lim C. (1983). Sporadic cases of hemolyticuremic syndrome associated with faecal cytotoxin and cytotoxinproducing *Escherichia coli* in stools. *Lancet*, 2:619–20.
4. Siegler R.L. (2003). Postdiarrheal Shiga toxin-mediated hemolytic uremic syndrome. *JAMA,* 290:1379–1381.
5. Brooks, J. T. *et al.* (2005). Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. *J. Infect. Dis.* https://doi.org/10.1086/466536.
6. Scallan, E. *et al.* (2011). Foodborne illness acquired in the United States—Major pathogens. *Emerg. Infect. Dis,*17: 7–15. https://doi.org/10.3201/eid1701.P11101.
7. Gould, L. H. *et al.* (2013). Increased recognition of non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States during 2000–2010: Epidemiologic features and comparison with *E. coli* O157 Infections. *Foodborne Pathog. Dis,* 10: 453–460. https://doi.org/10.1089/fpd.2012.1401.
8. Valilis, E., Ramsey, A., Sidiq, S. & DuPont, H. L. (2018). Non-O157 Shiga toxin-producing *Escherichia coli*-A poorly appreciated enteric pathogen: Systematic review. *Int. J. Infect. Dis,* 76: 82–87. https://doi.org/10.1016/j.ijid.2018.09.002.

9. Kaper, J., Nataro, J. & Mobley, H. (2004). Pathogenic *Escherichia coli*. *Nat Rev Microbiol,* 2: 123–140. https://doi- org.ezp2.lib.umn.edu/10.1038/nrmicro818
10. Melton-Celsa, A., Mohawk, K., Teel, L., & O'Brien, A. (2012). Pathogenesis of Shiga-toxin producing escherichia coli. *Current topics in microbiology and immunology*, *357*: 67–103. https://doi.org/10.1007/82_2011_176
11. Paton, J. C., & Paton, A. W. (1998). Pathogenesis and diagnosis of Shiga toxin-producing Escherichia coli infections. *Clinical microbiology reviews*, *11*(3), 450–479. https://doi.org/10.1128/CMR.11.3.450
12. Shaikh, N., & Tarr, P. I. (2003). Escherichia coli O157:H7 Shiga toxin-encoding bacteriophages: integrations, excisions, truncations, and evolutionary implications. *Journal of bacteriology*, *185*(12): 3596–3605. https://doi.org/10.1128/JB.185.12.3596-3605.2003
13. Rodríguez-Rubio, L., Haarmann, N., Schwidder, M., Muniesa, M., & Schmidt, H. (2021). Bacteriophages of Shiga Toxin-Producing *Escherichia coli* and Their Contribution to Pathogenicity. *Pathogens (Basel, Switzerland)*, *10*(4): 404. https://doi.org/10.3390/pathogens10040404
14. Melton-Celsa, A. R. (2014). Shiga toxin (Stx) classifcation, structure, and function. *Microbiol. Spectr.* https://doi.org/10.1128/microbiols pec.EHEC-0024-2013.
15. Fakhouri, F., Zuber, J., Frémeaux-Bacchi, V., & Loirat, C. (2017). Haemolytic uraemic syndrome. *The Lancet, 390*(10095):681-696. https://doi.org/10.1016/S0140-6736(17)30062-4

16. Kaplan, B. S., Meyers, K. E., & Schulman, S. L. (1998). The pathogenesis and treatment of hemolytic uremic syndrome. *Journal of the American Society of Nephrology : JASN*, *9*(6): 1126–1133. https://doi.org/10.1681/ASN.V961126

17. Byrne, L., Adams, N., & Jenkins, C. (2020). Association between Shiga Toxin-Producing Escherichia coli O157:H7 stx Gene Subtype and Disease Severity, England, 2009-2019. *Emerging infectious diseases*, *26*(10): 2394–2400. https://doi.org/10.3201/eid2610.200319

18. Centers for Disease Control and Prevention. (2021). E. coli (Escherichia coli) Symptoms. Retrieved from: https://www.cdc.gov/ecoli/ecoli-symptoms.html

19. Centers for Disease Control and Prevention. (2023). FoodNet Fast HUS. Retrieved from: https://wwwn.cdc.gov/FoodNetFast/HUS

20. Etcheverría, A. I., & Padola, N. L. (2013). Shiga toxin-producing Escherichia coli: factors involved in virulence and cattle colonization. *Virulence*, *4*(5), 366–372. https://doi.org/10.4161/viru.24642

21. Friedrich, A. W. et al. (2002). *Escherichia coli* harboring Shiga toxin 2 gene variants: Frequency and association with clinical symptoms. *J. Infect. Dis,* 185: 74–84. https://doi.org/10.1086/338115.

22. Corrigan, J. J. & Boineau, F. G. (2001). Hemolytic-Uremic Syndrome. *Pediatrics in Review, 22*(11): 365-369.

23. Pickering, L.K., Baker, C.J., Kimberlin, D.W., *Escherichia coli* Diarrhea In: American Academy of Pediatrics Red Book: 2012 Report of the Committee on Infectious Diseases, Elk Grove Village, IL, 2012, pp. 324-328.

24. Centers for Disease Control and Prevention (CDC). National Shiga toxin-producing Escherichia coli (STEC) Surveillance Overview. Atlanta, Georgia: US Department of Health and Human Services, CDC, 2012. Retrieved from: https://www.cdc.gov/ncezid/dfwed/pdfs/national-stec-surveillance-overiew-508c.pdf.

25. Centers for Disease Control and Prevention. (2018). National Enteric Disease Surveillance: Shiga Toxin-producing *Escherichia coli* (STEC) Annual Report, 2016. Retrieved from: https://www.cdc.gov/ecoli/surv2016/index.html

26. Centers for Disease Control and Prevention. (2018). National Outbreak Reporting System (NORS). Retrieved from: https://wwwn.cdc.gov/norsdashboard/

27. Centers for Disease Control and Prevention. (1982). Epidemiologic Notes and Reports: Isolation of *E. coli* O157:H7 from Sporadic Cases of Hemorrhagic Colitis—United States. *Morbidity and Mortality Weekly Report,* 31(43):580, 585.

28. University of Minnesota Extension. (2021). The ruminant digestive system. Retrieved from: https://extension.umn.edu/dairy-nutrition/ruminant-digestive-system. (accessed 25 October 2021).

29. Ferens, W.A., Hovde, C.J. (2014). Escherichia coli O157: H7: animal reservoir and sources of human infection. *Foodborne Pathogens and Disease*, 8: 465-487. doi: 10.1089/fpd.2010.0673

30. Friesema, I.H., Van De Kassteele, J., De Jager, C.M., et al. (2011). Geographical association between livestock density and human Shiga toxin-producing Escherichia coli O157 infections. *Epidemiol Infect*, *139*:1081–1087. doi: 10.1017/S0950268810002050.

31. Michel P., Wilson J.B., Martin S.W., et al. (1999). Temporal and geographical distributions of reported cases of Escherichia coli O157:H7 infection in Ontario. *Epidemiol Infect,* 122:193–200. doi: 10.1017/S0950268899002083.

32. Frank, C., Kapfhammer, S., Werber, D., et al. (2008). Cattle density and Shiga toxin-producing Escherichia coli infection in Germany: increased risk for most but not all serogroups. *Vector Borne Zoonotic Dis*, 8:635–643. doi: 10.1089/vbz.2007.0237.

33. Daly, R.F., House, J., Stanek, D., Stobierski, M. G. (2017). Compendium of measures to prevent disease associated with animals in public settings. *JAVMA, 25*(11).

34. Centers for Disease Control and Prevention. (2017). *E. coli (Escherichia coli)* Prevention. Retrieved from: https://www.cdc.gov/ecoli/ecoli-prevention.html

35. Adhikari, U., Esfahanian, E., Mitchell, J., et al. (2020). Quantitation of Risk Reduction of *E. coli* Transmission after Using Antimicrobial Hand Soap. *Pathogens*. 2020; 9(10):778. https://doi.org/10.3390/pathogens9100778

36. CDC. (2001). Outbreaks of Escherichia coli O157:H7 infections among children associated with farm visits—Pennsylvania and Washington, 2000. *MMWR Morb Mortal Wkly Rep*, 50:293–297.

37. Crump J.A., Sulka A.C., Langer A.J., et al. (2002). An outbreak of Escherichia coli O157:H7 infections among visitors to a dairy farm. *N Engl J Med*, 347:555–560.

38. Friedman C.R., Torigian C., Shillam P.J., et al. (1998). An outbreak of salmonellosis among children attending a reptile exhibit at a zoo. *J Pediatr,* 132:802–807.

39. CDC. (2005). Outbreaks of Escherichia coli O157:H7 associated with petting zoos—North Carolina, Florida, and Arizona, 2004 and 2005. *MMWR Morb Mortal Wkly Rep*, 54:1277– 1280.

40. Smith K.E., Stenzel S.A., Bender J.B., et al. (2004). Outbreaks of enteric infections caused by multiple pathogens associated with calves at a farm day camp. *Pediatr Infect Dis J*, 23:1098–1104.

41. Keen J.E., Elder, R.O. (2002). Isolation of Shiga-toxigenic Escherichia coli O157 from hide surfaces and the oral cavity of finished beef feedlot cattle. *J Am Vet Med Assoc*, 220:756–763.

42. Sayers G.M., Dillon M.C., Connolly E., et al. (1996). Cryptosporidiosis in children who visited an open farm. *Commun Dis Rep CDR Rev*, 6:R140–R144.

43. David S.T., MacDougall L., Louie K., et al. (2004). Petting zoo-associated Escherichia coli O157:H7—secondary transmission, asymptomatic infection, and prolonged shedding in the classroom. *Can Commun Dis Rep*, 30:173–180.

44. Crump J.A., Braden C.R., Dey M.E., et al. (2003). Outbreaks of Escherichia coli O157 infections at multiple county agricultural fairs: a hazard of mixing cattle, concessions stands and children. *Epidemiol Infect,* 131:1055–1062.

45. Curran K., Heiman K.E., Singh T., et al. (2015). Notes from the field: outbreak of Escherichia coli O157:H7 infections associated with dairy education event attendance—Whatcom county, Washington, 2015. *MMWR Morb Mortal Wkly Rep,* 64:1202–1203.

46. Payne, C.J.I, Petrovic, M., Roberts, R.J., et al. Vero cytotoxin–producing Escherichia coli O157 gastroenteritis in farm visitors, North Wales. Emerg Infect Dis 2003;9:526–530.

47. Loharikar A., Briere E., Schwensohn C., et al. (2012). Four multistate outbreaks of human Salmonella infections associated with live poultry contact, United States, 2009. *Zoonoses Public Health*, 59:347–354.

48. Hedican, E., Miller, B., Ziemer, B., et al. (2010). Salmonellosis outbreak due to chicken contact leading to a foodborne outbreak associated with infected delicatessen workers. *Foodborne Pathog Dis*, 7:995–997.

49. McMillian, M., Dunn, J.R., Keen, J.E., et al. (2007). Risk behaviors for disease transmission among petting zoo attendees. *J Am Vet Med Assoc,* 231:1036–1038.

50. Goode, B., O'Reilly, C., Dunn, J., et al. (2009). Outbreak of Escherichia coli O157:H7 infections after petting zoo visits, North Carolina state fair, October–November 2004. *Arch Pediatr Adolesc Med*,163:42–48.

51. Venegas-Vargas, C., Henderson, S., Khare, A., et al. (2016). Factors associated with Shiga toxin-producing *Escherichia coli* shedding by dairy and beef cattle. *Applied and Environmental Microbiology, 82*(16): 5049-56.

52. Mir, R. A., Weppelmann, T. A., Kanga, M., et al. (2014). Association between animal age and the prevalence of Shiga toxin-producing *Escherichia coli* in a cohort of beef cattle. *Vet. Microbiol,* 175, 325–331. doi: 10.1016/j.vetmic.2014.12.016

53. Ekiri, A.B., Landblom, D., Doetkott, D., et al. (2014). Isolation and characterization of Shiga toxin-producing *Escherichia coli* serogroups O26, O45, O103, O111, O113, O121, O145, and O157 shed from range and feedlot cattle from postweaning to slaughter. *Journal of Food Protection, 77*(7): 1052-1061.

54. Cáceres, M.E., Etcheverría, A.I., Fernández, D., et al. (2017). Variation in the Distribution of Putative Virulence and Colonization Factors in Shiga Toxin-Producing *Escherichia coli* Isolated from Different Categories of Cattle. *Front Cell Infect Microbiol,* 7:147. doi: 10.3389/fcimb.2017.00147. PMID: 28503491; PMCID: PMC5408013.

55. Bach, S.J., et al. (2004). Long-haul transport and lack of preconditioning increases fecal shedding of Escherichia coli and Escherichia coli O157:H7 by calves. *J Food Prot,* 67(4): 672-8.

56. Dunn, J.R., Keen, J.E., Thompson, R.A. (2004). Prevalence of Shiga-toxigenic Escherichia coli O157:H7 in adult dairy cattle. *J Am Vet Med Assoc*, 224:1151–1158. doi: 10.2460/javma.2004.224.1151.

57. Cobbold, R.N., Rice, D.H., Szymanski, M., Call, D.R., Hancock, D.D. (2004). Comparison of Shiga-toxigenic Escherichia coli prevalences among dairy, feedlot, and cow-calf herds in Washington State. *Appl Environ Microbiol*, 70:4375–4378. doi: 10.1128/AEM.70.7.4375-4378.2004.

58. Dewsbury, D.M., Renter, D.G., Shridhar, P.B., et al. (2015). Summer and winter prevalence of Shiga toxin-producing Escherichia coli (STEC) O26, O45, O103, O111, O121, O145, and O157 in feces of feedlot cattle. *Foodborne Pathog Dis*, 12:726–732. doi: 10.1089/fpd.2015.1987.

59. Jaros, P., Cookson, A. L., Reynolds, A., et al. (2018). The effect of transportation and lairage on faecal shedding and carcass contamination with *Escherichia coli* O157 and O26 in very young calves in New Zealand. *Epidemiology and Infection, 146*(9): 1089-1100.

60. Dewell, G.A., Simpson, C.A., Dewell, R.D. (2008). Impact of transportation and lairage on hide contamination with Escherichia coli O157 in finished beef cattle. *J. Food Prot,* 71:1114-1118.

61. Mather, A. E., G. T. Innocent, S. A., et al. (2007). Risk factors for hide contamination of Scottish cattle at slaughter with Escherichia coli O157. *Prev. Vet. Med,* 80:257-270.

62. Fukushima, H. Seki, R. (2004). High numbers of Shiga toxin-producing *Escherichia coli* found in bovine faeces collected at slaughter in Japan, *FEMS Microbiology Letters*, 238(1): 189–197. https://doi.org/10.1111/j.1574-6968.2004.tb09755.x

63. Omisakin, F., MacRae, M., Ogden, I.D., Strachan, N.J.C. (2003). Concentration and prevalence of *Escherichia coli* O157 in cattle feces at slaughter. *Applied and Environmental Microbiology, 69*(5), https://doi.org/10.1128/AEM.69.5.2444-2447.2003

64. Brownlie, L.E. & Grau, F.H. (1967). Effect of food intake on growth and survival of salmonellas and *Escherichia coli* in the bovine rumen. *J. Gen. Microbiol.*, *46* (1): 125-134.

65. Rostagno, M.H. (2009). Can stress in farm animals increase food safety risk? *Foodborne Pathogens and Disease, 6*(7). 10.1089=fpd.2009.0315

66. Mitchell, G., Hattingh, J., Ganhao, M. (1988). Stress in cattle assessed after handling, after transport and after slaughter. *The Veterinary Record,* 123(8):201-205. DOI: 10.1136/vr.123.8.201. PMID: 3176273.

67. Grandin, T. (1997). Assessment of stress during handling and transport. *Journal of Animal Science*, 75(1): 249–257. https://doi.org/10.2527/1997.751249x

68. Sperandio, V., Torres, A.G., Jarvis, B., et al. (2003). Bacteria-host communication: The language of hormones. *Biological Sciences, 100*(15): 8951-8956.

69. Webb, C.R. (2006). Investigating the potential spread of infectious diseases of sheep via agricultural shows in Great Britain. *Epidemiol Infect,* 134(1):31-40. doi: 10.1017/S095026880500467X. PMID: 16409648; PMCID: PMC2870361.

70. Ekong, P.S., Sanderson, M.W., Cernicchiaro, N. (2015). Prevalence and concentration of *Escherichia coli* O157 in different seasons and cattle types processed in North America:

A systematic review and meta-analysis of published research. *Preventive Veterinary Medicine, 121*(1-2): 74-85.

71. Stanford, K., Bach, S.J., Marx, T.H., et al. (2005). Monitoring *Escherichia coli* O157:H7 in inoculated and naturally colonized feedlot cattle and their environment. *J Food Prot*, *68*: 26-33.

72. Munns, K. D., Selinger, L. B., Stanford, K., et al. (2015). Perspectives on super-shedding of Escherichia coli O157:H7 by cattle. *Foodborne pathogens and disease*, *12*(2), 89–103. https://doi.org/10.1089/fpd.2014.1829

73. Chase-Topping, M. E., McKendrick, I. J., Pearce, M. C., et al. (2007). Risk factors for the presence of high-level shedders of Escherichia coli O157 on Scottish farms. *Journal of clinical microbiology*, *45*(5), 1594–1603. https://doi.org/10.1128/JCM.01690-06

74. Matthews, L., et al. (2006). Super-shedding cattle and the transmission dynamics of Escherichia coli O157. *Epidemiol Infect*, 134(1): 131-42

75. Grauke, L. J., Kudva, I. T., Yoon, J. W., et al. (2002). Gastrointestinal tract location of Escherichia coli O157:H7 in ruminants. *Applied and environmental microbiology*, *68*(5), 2269–2277. https://doi.org/10.1128/AEM.68.5.2269-2277.2002

76. Naylor, S. W., Low, J. C., Besser, T. E. (2003). Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic Escherichia coli O157:H7 in the bovine host. *Infection and immunity*, *71*(3), 1505–1512. https://doi.org/10.1128/IAI.71.3.1505-1512.2003

77. Cobbold, R. N., Hancock, D. D., Rice, D. H., et al. (2007). Rectoanal junction colonization of feedlot cattle by Escherichia coli O157:H7 and its association with supershedders and

excretion dynamics. *Applied and environmental microbiology*, *73*(5), 1563–1568. https://doi.org/10.1128/AEM.01742-06

78. Galland, J.C., Hyatt, D.R., Crupper, S.S., et al. (2001). Prevalence, antibiotic susceptibility, and diversity of Escherichia coli O157:H7 isolates from a longitudinal study of beef cattle feedlots. *Appl Environ Microbiol*. 67(4):1619-27. doi: 10.1128/AEM.67.4.1619-1627.2001.
79. Dewell, G.A., et al. (2005). Prevalence of and risk factors for Escherichia coli O157 in market-ready beef cattle from 12 U.S. feedlots. *Foodborne Pathog Dis*, 2(1): p. 70-6.
80. Jacob, M.E., et al., Feeding supplemental dried distiller's grains increases faecal shedding of Escherichia coli O157 in experimentally inoculated calves. Zoonoses Public Health, 2008. 55(3): p. 125-32.
81. Wells, J.E., et al. (2009). Prevalence and level of Escherichia coli O157:H7 in feces and on hides of feedlot steers fed diets with or without wet distillers grains with solubles. *J Food Prot,* 72(8): 1624-33.
82. Berry, E.D. & Wells, J.E. (2010). Escherichia coli O157:H7: recent advances in research on occurrence, transmission, and control in cattle and the production environment. *Adv Food Nutr Res*, 60: 67-117.
83. Jacob, M.E., Callaway, T.R., Nagaraja, T.G. (2009). Dietary interactions and interventions affecting Escherichia coli O157 colonization and shedding in cattle. *Foodborne Pathog Dis*, 6(7): 785-92.
84. Callaway, T.R., et al. (2011). Escherichia coli O157:H7 populations in ruminants can be reduced by orange peel product feeding. *J Food Prot*, 74(11): 1917-21.

85. Garber, L.P., et al. (1995). Risk factors for fecal shedding of Escherichia coli O157:H7 in dairy calves. *J Am Vet Med Assoc*, 207(1): 46-9.

86. Callaway, T.R., et al. (2013). Shiga Toxin-Producing Escherichia coli (STEC) Ecology in Cattle and Management Based Options for Reducing Fecal Shedding. *Agric. Food Anal. Bacteriol*, 3(1): 39-69.

87. Braden, K.W., et al. (2004). Ascophyllum nodosum supplementation: a preharvest intervention for reducing Escherichia coli O157:H7 and Salmonella spp. in feedlot steers. *J Food Prot*, 67(9): 1824-8.

88. Stephens, T.P., et al. (2007). Reduction of Escherichia coli O157 and Salmonella in feces and on hides of feedlot cattle using various doses of a direct-fed microbial. *J Food Prot*, 70(10): p. 2386-91.

89. Younts-Dahl, S.M., et al. (2004). Dietary supplementation with Lactobacillus- and Propionibacteriumbased direct-fed microbials and prevalence of Escherichia coli O157 in beef feedlot cattle and on hides at harvest. *J Food Prot*, 67(5): 889-93.

90. Schmidt, N., Barth, S.A., Frahm, J., et al. (2018). Decreased STEC shedding by cattle following passive and active vaccination based on recombinant *Escherichia coli* Shiga toxoids. *Veterinary Research, 49*:28. https://doi.org/10.1186/s13567-018-0523-0.

91. Kudva, I.T., Blanch, K., Hovde, C.J. (1998). Analysis of Escherichia coli O157:H7 survival in ovine or bovine manure and manure slurry. *Appl Environ Microbiol.* 64(9):3166-74. doi: 10.1128/AEM.64.9.3166-3174.1998.

92. Keen, J.E., Wittum, T.E., Dunn, J.R., et al. (2006). Shiga-toxigenic Escherichia coli O157 in agricultural fair livestock, United States. *Emerg Infect Dis,* 12(5):780-6. doi: 10.3201/eid1205.050984.

93. Varma, J.K., Greene, K.D., Reller, M.E., et al. (2003). An outbreak of Escherichia coli O157 infection following exposure to a contaminated building. *JAMA,* 290(20):2709-12. doi: 10.1001/jama.290.20.2709. PMID: 14645313.

94. Lanier, W.A., Hall, J.M., Herlihy, R.K., et al. (2011). Outbreak of Shiga-toxigenic Escherichia coli O157: H7 infections associated with rodeo attendance, Utah and Idaho, 2009. *Foodborne Pathog Dis*, 8:1131–1133.

95. Durso, L.M., Reynolds, K., Bauer, N., et al. (2005). Shiga-toxigenic Escherichia coli O157:H7 infections among livestock exhibitors and visitors at a Texas county fair. Vector Borne Zoonotic Dis 2005;5:193–201.

96. Centers for Disease Control and Prevention (CDC). (2012). Outbreak of Shiga toxin-producing Escherichia coli O111 infections associated with a correctional facility dairy - Colorado, 2010. *MMWR Morb Mortal Wkly Rep.* 61(9):149-52.

97. Bopp, D.J., Sauders, B.D., Waring, A.L., et al. (2003). Detection, isolation, and molecular subtyping of Escherichia coli O157:H7 and Campylobacter jejuni associated with a large waterborne outbreak. *J Clin Microbiol,* 41(1):174-80. doi: 10.1128/JCM.41.1.174-180.2003.

98. Centers for Disease Control and Prevention (CDC). (1999). Outbreak of Escherichia coli O157:H7 and Campylobacter among attendees of the Washington County Fair-New York, 1999. *MMWR Morb Mortal Wkly Rep,* 48(36):803-5.

99. Halsby, K.D., Walsh, A.L., Campbell, C., et al. (2014). Healthy animals, healthy people: zoonosis risk from animal contact in pet shops, a systematic review of the literature. *PLoS One,* 9:e89309.

100. Gautam, R., Bani-Yaghoub, M., Neill, W.H., et al. (2011). Modeling the effect of seasonal variation in ambient temperature on the transmission dynamics of a pathogen with a free-living stage: example of Escherichia coli O157:H7 in a dairy herd. *Prev Vet Med*, 102:10–21. doi: 10.1016/j.prevetmed.2011.06.008.

101. Vachon, M.S., Khalid, M., Tarr, G.A.M., Hedberg, C., Bro wn, J.A. (2020). Farm animal contact is associated with progression to Hemolytic uremic syndrome in patients with Shiga toxin-producing *Escherichia coli*—Indiana, 2012-2018. *One Health, 11*. https://doi.org/10.1016/j.onehlt.2020.100175

102. Reymond, D., Johnson, R. P., Karmali, M. A. et al. (1996). Neutralizing antibodies to *Escherichia coli* vero cytotoxin 1 and antibodies to O157 lipopolysaccharide in healthy farm family members and urban residents*, Journal of Clinical Microbiology, 34*(9): 2053-2057.

103. Wilson, J. B., Clarke, R. C., Renwick, S. A. et al. (1996). Vero cytotoxigenic *Escherichia coli* infection in dairy farm families. *The Journal of Infectious Diseases, 175*:1021-7.

104. Lebov, J., Grieger, K., Womack, D. et al. (2017). A framework for One Health research. *One Health, 3*, 44-50, https://doi.org/10.1016/j.onehlt.2017.03.004.

105. Centers for Disease Control and Prevention. Hemolytic Uremic Syndrome, Post-diarrheal (HUS) 1996 Case Definition. https://wwwn.cdc.gov/nndss/conditions/hemolytic-uremic-syndrome-post-diarrheal/case-definition/1996/. (accessed 8 November 2019).

106. Mody R.K., Luna-Gierke R.E., Jones T.F. et al. Infections in Pediatric Postdiarrheal Hemolytic Uremic Syndrome: Factors Associated With Identifying Shiga Toxin–

Producing *Escherichia coli*, *Arch Pediatr Adolesc Med. 166 (2012) 902-909.* https://doi.org/10.1001/archpediatrics.2012.471

107. Persad, A.K., LeJeune, J.T. Animal Reservoirs of Shiga Toxin-Producing Escherichia coli, Microbiology Spectrum, 2(2014). doi:10.1128/microbiolspec.EHEC-0027-2014

108. Hedican, E.B., Medus, C., Besser, J. et al. Characteristics of O157 versus non-O157 Shiga toxin-producing Escherichia coli infections in Minnesota, 2000-2006, Clinical Infectious Diseases. 49(2009) 358-364. https://doi.org/10.1086/600302

109. Karmali, MA. Infection by Shiga toxin-producing *Escherichia coli*: an overview, Molecular Biotechnology. 26(2004) 117-122.

110. Eklund M., Leino K., Siitonen A. Clinical Escherichia coli strains carrying stx genes: stx variants and stx-positive virulence profiles, Journal of Clinical Microbiology. 40(2002) 4585-4593. DOI: 10.1128/JCM.40.12.4585-4593.2002.

111. Tarr G.A.M., Stokowski T., Shringi S. et al. Contribution and Interaction of Shiga Toxin Genes to Escherichia coli O157:H7 Virulence, Toxins. 22(2019). DOI: 10.3390/toxins11100607.

112. Fuller, C., Pellina, C., Flagler, M., Strasser, J., Weiss, A. Shiga toxin subtypes display dramatic differences in potency, Infection and Immunity. 79(2010) 1329-1337. DOI: 10.1128/IAI.01182-10.

113. United States Department of Agriculture. Indiana State and County Data Census of Agriculture, 2017. https://www.nass.usda.gov/Publications/AgCensus/2017/Full_Report/Volume_1,_Chapter_1_State_Level/Indiana/inv1.pdf, 2017 (accessed 22 September 2020).

114. Cho S., Fossler C.P., Diez-Gonzalez F. et al. Herd-level risk factors associated with fecal shedding of Shiga toxin-encoding bacteria on dairy farms in Minnesota, USA, *Can Vet J*. 54 (2013) 693-697.

115. Indiana State Department of Health. 2015 Communicable Disease Reporting Rule. https://www.in.gov/isdh/25366.htm. (accessed 9 November 2019).

116. Centers for Disease Control and Prevention. Shiga Toxin-producing Escherichia coli (STEC) 2006 Case Definition. https://wwwn.cdc.gov/nndss/conditions/shiga-toxin-producing-escherichia-coli/case-definition/2006/. (accessed 15 November 2019).

117. Centers for Disease Control and Prevention. (2014). Shiga Toxin-producing Escherichia coli (STEC) 2014 Case Definition. https://wwwn.cdc.gov/nndss/conditions/shiga-toxin-producing-escherichia-coli/case-definition/2014/. (accessed 15 November 2019).

118. Centers for Disease Control and Prevention. (2018). Shiga Toxin-producing *Escherichia coli* (STEC) 2018 Case Definition. Available at: https://wwwn.cdc.gov/nndss/conditions/shiga-toxin-producing-escherichia-coli/case-definition/2018/. Accessed 15 November 2019.

119. Imai, K., Tingley, D., Keele, L. A general approach to causal mediation analysis, Psychological Methods. 15(2010) 309-334. DOI: 10.1037/a0020761.

120. Bettelheim, K. The Non-O157 Shiga-Toxigenic (Verocytotoxigenic) Escherichia coli; under-rated pathogens, Critical Reviews in Microbiology. 33 (2007) 67-87. DOI: 10.1080/10408410601172172.

121. O'Brien, S., Adak, G., Gilham, C. Contact with farming environment as a major risk factor for Shiga toxin (vero cytotoxin)-producing Escherichia coli O157 infection in humans, Dispatches. 7(2001) 1049-1051. doi: 10.3201/eid0706.010626.

122. Ardissino G., Tel F., Testa S. et al. A simple prognostic index for Shiga toxin-related hemolytic uremic syndrome at onset: data from the ItalKid-HUS network, Eur J Pediatr. 177 (2018) 1667–1674. DOI:10.1007/s00431-018-3198-7.

123. McKee, R. Schnadower, D., Tarr, P. et al. Predicting Hemolytic Uremic Syndrome and Renal Replacement Therapy in Shiga Toxin–producing *Escherichia coli*–infected Children, *Clinical Infectious Diseases*. 70(2020). DOI: https://doi.org/10.1093/cid/ciz432

124. Majkowski J. Strategies for rapid response to emerging foodborne microbial hazards, Emerging Infectious Diseases. 3(1997) 551–554. doi: 10.3201/eid0304.970420

125. Katani, R. Cote, R., Kudva, I. et al. Comparative genomics of two super-shedder isolates of Escherichia coli O157:H7, PLoS One. 12(2017). doi: 10.1371/journal.pone.0182940

126. Stein, R. Katz, D. Escherichia coli, cattle and the propagation of disease. FEMS Microbiology Letters. 364 (2017). https://doi.org/10.1093/femsle/fnx050.

127. Blanco, M., Blanco, J.E., Mora, A. et al. Serotypes, virulence genes, and intimin types of Shiga toxin (Verotoxin)-producing Escherichia coli isolates from cattle in Spain and identification of a new intimin variant gene (eae-), Journal of Clinical Microbiology. 44(2004) 645-651. DOI: 10.1128/JCM.42.2.645–651.2004.

128. Franz, E. van Hoek, A. H. A. M., van der Wal, F. J. et al. Genetic features differentiating bovine, food, and human isolates of Shiga toxin-producing *Escherichia coli* O157 in the Netherlands, Journal of Clinical Microbiology. 50(2011) 772-780. doi: 10.1128/JCM.05964-11

129. Kudva, I., Hatfield, P., Hovde, C. Characterization of *Escherichia coli* O157:H7 and other Shiga toxin-producing E. coli serotypes Isolated from sheep, Journal of Clinical Microbiology. 35(1997) 892-899.

130. Donnenberg, M., Tzipori, S., McKee, M. et al. The role of the *eae* gene of the enterohemorrhagic Escherichia coli in intimate attachment in vitro and in a porcine model, The Journal of Clinical Investigation. 92(1993) 1418-1424. https://doi.org/10.1172/JCI116718.

131. Persson, S. Olsen, K., Ethelberg, S. et al. Subytping method for Escherichia coli Shiga toxin (Verocytotoxin) 2 variants and correlations to clinical manifestations, Journal of Clinical Microbiology. 45(2006) 2020-2024. **DOI:** 10.1128/JCM.02591-06

132. Thorpe, C.M. Shiga toxin—producing *Escherichia coli* infection, *Clinical Infectious Diseases. 38 (2004)* 1298–1303. DOI: https://doi.org/10.1086/383473.

133. Wong, C.S., Jelacic S., Habeeb, R.L. et al. The risk of the hemolytic-uremic syndrome after antibiotic treatment of Escherichia coli O157: H7 infections, N Engl J Med. 342(2000)1930–1936. doi: 10.1056/NEJM200006293422601.

134. Tarr, G., Oltean, H. N., Phipps, A. I. et al. Case definitions of hemolytic uremic syndrome following Escherichia coli O157:H7 infection vary in validity, International Journal of Medical Microbiology. 308(2018) 1121–1127. https://doi.org/10.1016/j.ijmm.2018.10.002.

135. Hunt, J. M. (2010). Shiga toxin-producing *Escherichia coli* (STEC). *Clinics in Laboratory Medicine, 30*(1). https://doi.org/10.1016/j.cll.2009.11.001

136. Kassenborg H.D., Hedberg C.W., Hoekstra M., et al. (2004). Farm visits and undercooked hamburgers as major risk factors for sporadic *Escherichia coli* O157:H7 infection: data from a case-control study in 5 FoodNet sites. *Clin Infect Dis, 38*(Suppl3): S271-278.

137. Voetsch, A.C., Kennedy M.H., Keene W.E., et al. (2007). Risk factors for sporadic Shiga toxin-producing *Escherichia coli* O157 infections in FoodNet Sites, 1999–2000*. Epidemiol Infect,135*(6):993–1000.

138. Minnesota Department of Health. (2019). Reporting Enteric *Escherichia coli* Infection. Retrieved from: https://www.health.state.mn.us/diseases/ecoli/report.html#what

139. Centers for Disease Control and Prevention. (2021). National Notifiable Diseases Surveillance System (NNDSS) Shiga toxin-producing *Escherichia coli* (STEC). Retrieved from: https://ndc.services.cdc.gov/conditions/shiga-toxin-producing-escherichia-coli/

140. Centers for Disease Control and Prevention. (2021). National Notifiable Diseases Surveillance System (NNDSS) Hemolytic Uremic Syndrome, Post-diarrheal (HUS). Retrieved from: https://ndc.services.cdc.gov/conditions/hemolytic-uremic-syndrome-post-diarrheal/

141. Minnesota Department of Health. (2022). Reporting Hemolytic Uremic Syndrome. Retrieved from: https://www.health.state.mn.us/diseases/ecoli/reporthus.html

142. Ethelberg S, Olsen KE, Scheutz F, Jensen C, Schiellerup P, Enberg J, Petersen AM, Olesen B, Gerner-Smidt P, Mølbak K. Virulence factors for hemolytic uremic syndrome, Denmark. Emerg Infect Dis. 2004 May;10(5):842-7. doi: 10.3201/eid1005.030576. PMID: 15200817; PMCID: PMC3323205.

143. Byrne, L., Jenkins, C., Launders, N., et al. (2015). The epidemiology, microbiology and clinical impact of Shiga toxin-producing *Escherichia coli* in England, 2009-2012. *Epidemiology and Infection, 143I*(16), 3475-3487. doi:10.1017/S0950268815000746

144. Cleary E., Boudou M., Garvey P., et al. (2021). Spatiotemporal dynamics of sporadic Shiga toxin-producing *Escherichia coli* enteritis, Ireland, 2013-2017. *Emerg Infect Dis. 27*(9):2421-2433. doi: 10.3201/eid2709.204021.

145. United States Department of Agriculture. (2017). 2017 Census of Agriculture. USDA, National Agricultural Statistics Service. Retrieved from: https://www.nass.usda.gov/Publications/AgCensus/2017/index.php

146. Minnesota State Demographic Center Department of Administration. (2021). PopFinder for Minnesota, Counties, & Regions. Retrieved from: https://mn.gov/admin/demography/data-by-topic/population-data/our-estimates/pop-finder1.jsp

147. Van Buuren, S., Groothuis-Oudshoorn, K. (2011). Mice: multivariate imputation by chained equations in R. *J Stat Softw,45*:1–67.

148. Rubin, DB. Multiple imputation for nonresponse in surveys. New York, NY: John Wiley & Sons, 1987.

149. Locking M., Allison L., Rae L., et al. (2006). VTEC infections and livestock-related exposures in Scotland, *Euro Surveill, 11*(8): pii=2908. https://doi.org/10.2807/esw.11.08.02908-en

150. Schlager, S., Lepuschitz, S., Ruppitsch, W., et al. (2018). Petting zoos as sources of Shiga toxin-producing *Escherichia coli* (STEC) infections. *International Journal of Medical Microbiology, 308*(7): 927-932.

151. Tack D.M., Kisselburgh H.M., Richardson L.C., et al. (2021). Shiga toxin producing *Escherichia coli* outbreaks in the United States, 2010-2017. *Microorganisms, 9*(7): 1529. https://doi.org/10.3390/microorganisms9071529

152. Corrier, D. E., Purdy, C. W., & DeLoach, J. R. (1990). Effects of marketing stress on fecal excretion of Salmonella spp in feeder calves. *American journal of veterinary research*, *51*(6), 866–869.

153. Stroebel, C., Alexander, T. Workentine, M.L., Timsit, E. (2018). Effects of transportation to and co-mingling at an auction market on nasopharyngeal and tracheal bacterial communities of recently weaned beef cattle. *Veterinary Microbiology, 223*:126-133. https://doi.org/10.1016/j.vetmic.2018.08.007.

154. Erdozain G, KuKanich K, Chapman B, et al. (2013). Observation of public health risk behaviours, risk communication and hand hygiene at Kansas and Missouri petting zoos—2010–2011. *Zoonoses Public Health*,*60*:304–310.

155. Weese JS, McCarthy L, Mossop M., et al. (2007). Observation of practices at petting zoos and the potential impact on zoonotic disease transmission. ***Clin Infect Dis***, *45*:10–15.

156. Davis, M.A., Sheng, H., Hewman, J., et al. (2006). Comparison of a waterless hand hygiene preparation and soap-and-water hand washing to reduce coliforms on hands in animal exhibit settings. *Epidemiol Infect*, *134*:1024-1028.

157. LeJeune, J.T., Davis, M.A. (2004). Outbreaks of zoonotic enteric disease associated with animal exhibits. *J Am Vet Med Assoc. 224*: 1440-1445.

158. Centers for Disease Control and Prevention. Handwashing: clean hands save lives. Show me the science—when & how to use hand sanitizer. Available at: www. cdc.gov/handwashing/show-me-the-science-hand-sanitizer. html. Accessed January 24, 2022.

159. Ibarra M., Meehan C., Daniels M., et al. (2022). Low prevalence of handwashing and importance of signage at California county fair animal exhibits. *Calif Agr 75*(3):151-155. https://doi.org/10.3733/ca.2021a0015.

160. McIntosh, E. (2006). Using Discrete Choice Experiments within a Cost-Benefit Analysis Framework. *Pharmacoeconomics 24*: 855-868. https://doi.org/10.2165/00019053-200624090-00004

161. Pragle, A. S. Harding, A. K., Mack, J. C. (2007). Food workers' perspectives on handwashing behaviors and barriers in the restaurant environment. *Journal of Environmental Health, 69*(10): 27-33.

162. To, K.G., Lee, J., Nam, Y., et al. (2016). Hand washing behavior and associated factors in Vietnam based on the Multiple Indicator Cluster Survey, 2010–2011. *Global Health Action, 9*:1, 29207. doi: 10.3402/gha.v9.29207

163. Graves, J., Daniell, W. E., Harris, J.R., et al. (2012). Enhancing a safe water intervention with student-created visual aids to promote handwashing behavior in

Kenyan primary schools. *Int'l. Quarterly of Community Health Education, 32*(4): 307-323. doi: http://dx.doi.org/10.2190/IQ.32.4.d

164. Biswas, D., Nizame, F., Sanghvi, T. et al. (2017). Provision versus promotion to develop a handwashing station: the effect on desired handwashing behavior. *BMC Public Health, 17*: 390. DOI 10.1186/s12889-017-4316-6

165. Blum, L. Yemweni, A., Trinies, V. et al., (2019). Programmatic implications for promotion of handwashing behavior in an internally displaced persons camp in North Kivu, Democratic Republic of Congo. *Conflict and Health, 13*: 54. https://doi.org/10.1186/s13031-019-0225-x

166. Contzen, N., Meili, I. H., Mosler, H. (2015). Changing handwashing behavior in southern Ethiopia: A longitudinal study on infrastructural and commitment interventions. *Social Science & Medicine, 124*: 103-114.

167. Chittleborough, C. R., Nicholson, A., Basker, E., et al. (2013). Factors influencing handwashing behavior in primary schools. *Health Educ Res. 27*(6): 1055-1068.

168. Borchgrevink, C. P., Cha, J., Kim, S. (2013). Handwashing practices in a college town environment. *Journal of Environmental Health, 75*(8): 18-25.

169. Johnson, H.D., Sholcosky, D., Gabello, K., et al. (2003). Sex differences in public restroom handwashing behavior associated with visual behavior prompts. *Perceptual and Motor Skills, 97*: 805-810.

170. Burusnukul, P., Broz, Charles Christopher. (2013). Drivers and motivators in consumer handwashing behavior. *Nutrition & Food Science, 43*(6): 596-604.

171. Ram, P., Halder, A., Granger, S. et al. (2010). Is structured observation a valid technique to measure handwashing behavior? Use of acceleration sensors embedded in soap to assess reactivity to structured observation. *Am. J. Trop. Med. Hyg., 83*(5): 1070-1076.

172. Jeong, J.S., Choi, J. K., Jeong, I.S., et al. (2007). A Nationwide survey on the handwashing behavior and awareness. *J Prev Med Public Health, 40*(3): 197-204.

173. Edwards, D., Monk-turner, E., Poorman, S., et al. (2002). Predictors of handwashing behavior. *Social Behavior and Personality, 30*(8): 751-756. doi 10.2224/sbp.2002.30.8.751

174. Galiani, S., Gertler, P., Ajzenman, N., Orsola-Vidal, A. (2016). Promoting handwashing behavior: the effects of large-scale community and school-level interventions. *Health Economics, 25*:1545-1559.

175. Edwards, D., Monk-turner, E., Poorman, S., et al. (2002). Predictors of handwashing behavior. *Social Behavior and Personality, 30*(8): 751-756. doi 10.2224/sbp.2002.30.8.751

176. Kuhfeld, W.F. (2010). Discrete Choice. Retrieved from: http://support.sas.com/techsup/technote/mr2010f.pdf

177. Johnson F.R., Kanninen B., Bingham M., Özdemir S. (2006) Experimental design for stated-choice studies. In: Kanninen B.J. (eds) Valuing Environmental Amenities Using Stated Choice Studies. The Economics of Non-Market Goods and Resources, vol 8. Springer, Dordrecht. https://doi.org/10.1007/1-4020-5313-4_7

178. University of Minnesota. (2017). D2D: The Driven to Discover Research Facility. Retrieved from: http://d2d.umn.edu/

179. McFadden D. Conditional logit analysis of qualitative choice behavior. Berkeley, CA: University of California; 1974.

180. Hauber AB, González JM, Groothuis-Oudshoorn CGM, Prior T, Marshall DA, Cunningham C, et al. Statistical methods for the analysis of discrete choice experiments: a report of the ISPOR Conjoint Analysis Good Research Practices Task Force. Value Heal [Internet]. 2016, 19:300-15 https://www.ispor.org/Conjoint-Analysis-Statistical-Methods-Guidelines.pdf.

181. Train, K. Discrete Choice Methods with Simulation. Cambridge University Press, 2009

182. Lancsar, E., Fiebig, D.G. & Hole, A.R. Discrete Choice Experiments: A Guide to Model Specification, Estimation and Software. *PharmacoEconomics* **35,** 697–716 (2017). https://doi.org/10.1007/s40273-017-0506-4

183. Anderson ME, Weese JS. (2012). Video observation of hand hygiene practices at a petting zoo and the impact of hand hygiene interventions. *Epidemiol Infect*, *140*:182–190. 207.

184. Pickering, A.J., Blum, A.G., Breiman, R.F., et al. (2014). Video surveillance captures student hand hygiene behavior, reactivity to observation, and peer influence in Kenyan primary schools. *PLoS ONE*

185. Cardinale Lagomarsino, B., Gutman, M., Freira, L., et al. (2017). Peer pressure: Experimental evidence from restroom behavior. *Economic Inquiry*, 55, 1579– 1584.

186. Grover, E., Hossain, M. K., Uddin, S., et al. (2018). Social Influence on Handwashing with Soap: Results from a Cluster Randomized Controlled Trial in Bangladesh. *The*

American journal of tropical medicine and hygiene, *99*(4), 934–936. https://doi.org/10.4269/ajtmh.17-0903

187. Minnesota State Fair. Attendance. Retrieved from: https://www.mnstatefair.org/about-the-fair/attendance/

188. Chirase , N.K., Greene, L.W., Purdy C.W. et al. (2004). Effect of transport stress on respiratory disease, serum antioxidant status, and serum concentrations of lipid peroxidation biomarkers in beef cattle. *Am J Vet Res, 65*(6):860-4. doi:10.2460/ajvr.2004.65.860. PMID: 15198229.

189. Krehbiel, C. R. (2020). Bovine respiratory disease influences on nutrition and nutrient metabolism. *Veterinary Clinics: Food Animal Practice, 36*: 361-373. doi: 10.1016/j.cvfa.2020.03.010

190. Burdick, N.C., Randel, R. D., Carroll, J.A., et al. (2011). Interactions between temperament, stress, and immune function in cattle. *International Journal of Zoology, 2011.* https://doi.org/10.1155/2011/373197

191. Arthington, J.D., Eichert, S. D., Kunkle, W.E., et al. (2003). Effect of transportation and commingling on the acute-phase protein response, growth, and feed intake of newly weaned beef calves. *J Anim Sci, 81,* 1120-1125.

192. Hong, H., Lee, E., Lee, I.H., et al. (2019). Effects of transport stress on physiological responses and milk production in lactating dairy cows. *Asian-Australas J Anim Sci*., *32*(3): 442-451. doi:105713/ajas.18.0108.

193. Dobson, H., Smith, R.F. (1995). Stress and reproduction in farm animals. *Journal of Reproduction and Fertility Supplement, 49*:451-461.

194. Girma, F., Gebremariam, B. (2019). Review on effect of stress on production and reproduction of dairy cattle. *Journal of Scientific and Innovative Research, 8*(1): 29-32.

195. Hultgren, J., Segerkvist, K.A., Berg, C., et al. (2022). Preslaughter stress and beef quality in relation to slaughter transport of cattle. *Livestock Science, 264*. https://doi.org/10.1016/j.livsci.2022.105073.

196. Whittlestone, W.G., Kilgour, R., De Langen, M. (1970). Behavioral stress and the cell count of bovine milk. *Journal of Milk and Food Technology, 33*:217-220.

197. Johnson, H.D. & Vanjonack, W.J. (1975). Symposium: Stress and health of the dairy cow. Effects of environmental and other stressors on blood hormone patterns in lactating animals. Department of Dairy Husbandry Animal Science Research Center University of Missouri Columbia 5201. Contribution from Missouri Agricultural Experiment Station Journal No. 7239.

198. Mechie, S.C., Chapman, P.A., Siddons, C.A. (1997). A fifteen month study of *Escherichia coli* O157:H7 in a dairy herd. *Epidemiol Infect., 118*:17-25. 10.1017/S0950268896007194.

199. Crookshank, H.R., Elissalde, M.H., White, R.G., et al., (1979). Effect of transportation and handling of calves upon blood serum composition. *Journal of Animal Science, 48*(3): 430-435.

200. Van Engen, N.K. & Coetzee, J.F. (2018). Effects of transportation on cattle health and production: a review. *Anim Health Res Rev., 19*(2): 142-154. doi: 10.1017/S1466252318000075.

201. Minnesota Future Farmers of America Association. (2022). Premier leadership, personal growth, and career success through agricultural education. Retrieved from: https://www.mnffa.org/

202. Minnesota Dairy Initiative. (2023). Tomorrow's way to dairy. Retrieved from: https://mn-dairy-initiative.org/

203. Medicus Health. (2022). Variable speed centrifuge, 6 place. Retrieved from: https://www.medicus-health.com/lab-supplies/laboratory-centrifuges/centrifuges-test-tubes-3-5-ml/variable-speed-centrifuge.html?gclid=CjwKCAjw682TBhATEiwA9crl38syJTRZHm9XxTGxbCIOdKBBPkAG3y9jSQ0j6gr4lfd5exnBndfDkxoCBE0QAvD_BwE

204. Virginia Tech. (2017). SOP: Blood Collection in Cattle. Retrieved from: https://ouv.vt.edu/content/dam/ouv_vt_edu/sops/large-animal/sop-bovine-blood-collection.pdf

205. McGill University. (2018). Macdonald Campus Farm Cattle Complex Standard Operating Procedure # DC-702. Retrieved from: https://www.mcgill.ca/research/files/research/dc702_collection_of_blood_sample.pdf.

206. Edwards, S., Evans, P., Hucklebridge, F., Clow, A. (2001). Association between time of awakening and diurnal cortisol secretory activity. *Psychoneuroendocrinology, 26*(6): 613-622.

207. Hudson, S., Whittlestone, W.G., Payne, E., (1975). Diurnal variations in blood cortisol in the dairy cow. *Journal of Dairy Science, 58*(1): 30-33.

208. Alila-Johansson, A., Eriksson, L., Soveri, T., et al. (2003). Serum cortisol levels I goats exhibit seasonal but not daily rhythmicity. *The Journal of Biological and Medical Rhythm Research, 20*(1): 65-79.

209. Domenech-Coca, C., Mariné-Casadó, R., Caimari, A., et al. (2019). Dual liquid-liquid extraction followed by LC-MS/MS method for the simultaneous quantification of melatonin, cortisol, triiodothyronine, thyroxine and testosterone levels in serum: Applications to a photoperiod study in rats. *J Chromatogr B Analyt Technol Biomed Life Sci.*, 1108:11-16. doi: 10.1016/j.jchromb.2019.01.002.

210. Nikolic, J.A., Begovic, J., Krsmanovic, J., et al. (1998). Basal serum cortisol concentrations in cattle. *Acta veterinaria, 48*: 265-275.

211. Fazio, E., Medica, P., Alberghina, D., et al. (2005). Effect of long-distance road transport on thyroid and adrenal function and hematocrit values in Limousin cattle: Influence of body weight decrease. *Veterinary Research Communications, 29*: 713-719.

212. Wernicki, A., Urban-chmiel, R., Kankofer, M., et al. (2006). Evaluation of plasma cortisol and TBARS levels in calves after short-term transportation. *Revue Méd. Vét, 157*(1): 30-34.

213. Tume, R.K. & Shaw, F.D. (1992). Beta-endorphin and cortisol concentrations in plasma of blood samples collected during exsanguination of cattle. *Meat Sciences, 31*: 211-217.

214. Probst, J.K., Neff, A.S., Hillmann, E., et al. (2014). Relationship between stress-related exsanguination blood variables, vocalization, and stressors imposed on cattle between lairage and stunning box under conventional abattoir conditions. *Livestock Science, 164*:154-158.

215. Beach, J.C., Murano, E.A., Acuff, G.R. (2002). Prevalence of *Salmonella* and *Campylobacter* in beef cattle from transport to slaughter. *Journal of Food Protection 65*(11):1687-1693.